Effect of Prolonged Stimulation of the Heme Oxygenase/Carbon Monoxide System by Hemin on Blood Pressure and Penile Erection of Spontaneously Hypertensive Rats

A Thesis

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for the Degree of
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By

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Abstract

Essential hypertension (EH) is a risk factor for many cardiovascular disorders. Treatment of established EH, especially for prolonged control of this pathogenic process, represents a great challenge. Moreover, hypertension is considered an important risk factor for the development of many other diseases, e.g. erectile dysfunction.

Hemin and other heme derivatives, e.g. heme-L-lysinate (HLL) and heme-L-arginate, have been used extensively to upregulate expression of heme oxygenase (HO) and production of endogenous carbon monoxide (CO). Short-term hemin administration for 4-5 days has been shown to markedly decrease high blood pressure (BP) in spontaneously hypertensive rats (SHR), but not in normotensive Wistar-Kyoto (WKY) or Sprague Dawley (SD) rats. This short-term therapy was effective in treating young, but not adult SHR. In the present study, hemin (15 mg/kg/day) was administered to 12-week old adult SHR through subcutaneously implanted osmotic minipumps for 3 consecutive weeks (the hemin protocol). Into the second week of the hemin protocol, BP of SHR was normalized from 203.2 ± 2.5 to 123.4 ±1.9 mmHg ($n=20$, $p<0.001$). There was no further decrease of BP in the remaining days of the hemin protocol. Normalization of BP in these treated SHR was maintained for 9 months after the removal of hemin pumps.

To further investigate the metabolic characteristics of hemin during hemin protocol administration, we attempted to monitor circulatory heme levels. A valid standard calibration curve was established using hemin or HLL in *in vitro* experiments. It was established that the basal serum level of heme was negligible in all rat strains prior to hemin protocol initiation. During the hemin protocol, serum heme level of all

rats was significantly elevated; however, it quickly dropped to basal levels thereafter.

At the end of the 3-week hemin protocol, HO-1 expression, HO activity, soluble guanylyl cyclase (sGC) expression, and cyclic guanosine monophosphate (cGMP) content were all increased, but phosphodiesterase-5 (PDE-5) expression was down-regulated in the mesenteric arteries. The hemin protocol also reversed SHR's decreased arterial lumen size, and increased expression levels of vascular endothelial growth factor. These changes lasted 9 months after the hemin protocol. The hemin protocol did not cause hepatic or renal toxicity. Our study thus unmasks a novel hemin protocol that will not only normalize BP in SHR with established hypertension but, more importantly, also provide long-lasting anti-hypertension protection. Sustained upregulation of the HO-regulated signaling pathways and reversal of vascular remodeling in small peripheral vessels in treated SHR are among potential underlying mechanisms for the anti-hypertensive effect of the hemin protocol.

We further studied if the beneficial effects of hemin protocol on BP of SHR could be extended to improvement of their penile erection. Intracavernosal pressure changes after electrical stimulation of the cavernous nerve (CN) were monitored in adult SHR and age-matched normotensive SD rats after administration of either hemin or hydralazine. Expression levels of HO-1, HO-2, sGC, and PDE-5 in penile tissues were also examined. Frequency-dependent intracavernosal pressure (ICP) changes were reduced in adult SHR. Three weeks after hemin treatment, ICP responses to CN stimulations were significantly increased to the level of normotensive rats, which was linked to normalization of BP of hemin-treated SHR. Hydralazine-treated SHR experienced normalization of BP but not ICP after 3 weeks of treatment. Expression of

HO-1 and sGC was upregulated and that of PDE-5 downregulated in hemin-treated SHR. Decreased ICP response in adult SHR can be improved through chronic hemin treatment of SHR. Prolonged upregulation of HO-1 and sGC as well as lowered expression of PDE-5 may account for normalization of erectile dysfunction in SHR subsequent to hemin treatment.

This thesis reports for the first time that 21-day hemin administration led to a 9-month normalization of high BP of adult SHR. These effects were mediated through sustained stimulation of the HO/CO system and its downstream effectors targets. Increased activity of HO-1 led to normalization of the abnormally high expression level of VEGF in peripheral mesenteric arteries of adult SHR. Subsequently, this resulted in reversal of the eutrophic remodeling of these arteries, which seems to be the priniciple determinant of the long-term normalization of BP observed for 9 months after stoppage of hemin treatment. The invention of hemin protocol offers a new therapeutic approach for the clinical management of established hypertension for a long duration.

Our study, for the first time, correlated changes in serum heme levels with BP levels after injection of hemin or HLL in normotensive and hypertensive rats. Application of this heme monitoring technology will also pave the way for clinical application of hemin therapy in treatment of EH.

Another intriguing finding in this thesis is that upregulation of HO-1, through the hemin protocol, led to improvement of abnormally low ICP encountered in adult SHR. Upregulation of HO-1 may represent a novel therapeutic approach to treat hypertension-related erectile dysfunction.

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DEDICATION

To my family

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

γ-glutamyltranspeptidase	(γGT)
4-phenylimidazole	(4-PD)
Acetylcholine	(ACh)
Acute intermittent porphyria	(AIP)
ALA synthase	(ALA-S)
Alanine aminotransferase	(ALT)
Angiotensin converting enzyme	(ACE)
Angiotensin II	(Ang-II)
Angiotensin-1	(AT1)
BP	(BP)
Calcium-activated potassium channels	(k_{ca})
Carbon monoxide	(CO)
Cavernosal arterial inflow	(CAI)
Coenzyme A	(CoA)
Complementary deoxyribonucleic acid	(cDNA)
Corporal veno-occlusive dysfunction	(CVOD)
Cyclic adenosine monophosphate	(cAMP)
Cyclooxygenase	(COX)
Cytochrome P450 monooxygenases	(CYP450)
Cytosolic hemebinding proteins	(CBP)
Dimethyl sulfoxide	(DMSO)
Endothelin-1	(ET-1)
Enodothelial NOS	(eNOS)

Endothelium-derived hyperpolarizing factor	(EDHF)
Erectile dysfunction	(ED)
Ethylenediaminetetraacetic acid	(EDTA)
H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1one	(ODQ)
Heme-binding proteins	(HeBPs)
Heme carrier protein	(HCP1)
Heme oxygenase-1	(HO-1)
Heme-L-lysinate	(HLL)
Hemoglobin	(Hb)
High performance liquid chromatography	(HPLC)
Inducible NOS	(iNOS)
Intracavernosal pressure	(ICP)
Isobutylmethyl xanthine	(IBMX)
Massachussets Male Aging Study	(MMAS)
Mean arterial BP	(MAP)
Messenger ribonuceliec acid	(mRNA)
Monocyte chemoattractant protein-1	(MCP-1)
Murine erythroleukemia	(MEL)
Nitric Oxide	(NO)
Nitric oxide synthase	(NOS)
Phenylephrine	(PHE)
Phosphodiesterase5	(PDE5)
Platelet rich plasma	(PRP)

Porphobilinogen	(PBG)
Potassium chloride	(KCl)
Reduced nicotinamide adenine dinucleotide	(NADH)
Reduced nicotinamide adenine dinucleotide phosphate	(NADPH)
S-nitroso-N-penicillamine	(SNAP)
Sodium nitroprusside	(SNP)
Soluble guanylate cyclase	(sGC)
Sparague-Dawley	(SD)
Spontaneously hypertensive rat	(SHR)
Stannous chloride	($SnCl_2$)
Tin protoporphyrin IX	(SnPPIX)
Vascular endothelial growth factor	(VEGF)
Vascular smooth muscle cells	(VSMC)
Wistar-Kyoto	(WKY)
Zinc protoporphyrin	(ZnPP)
Δ-aminolevulinic acid	(ALA)

LIST OF CHEMICALS AND ANTIBODIES

The following chemicals and antibodies were obtained as shown in the list below:

Hemin	Sigma
Heme-L-lysinate	Color Your Enzyme
Pyridine spectrophotometric reagent (99%)	Sigma
Sodium dithionite	Sigma
Anti sGC antibody	US Biologicals
Anti PDE5 antibody	EMD Biosciences
Anti iNOS antibody	StressGen
Anti eNOS antibody	StressGen
Anti HO-1 antibody	Affinity Bioreagents
Anti HO-2 antibody	StressGen
Anti VEGF-A antibody	Novus Biologicals
Total Bilirubin Kit	Diagnostics Chemicals Limited
^{125}I-cGMP-RIA	Amersham International

1. *General Introduction*

1.1 Heme

1.1.1 Introduction

Heme is a complex of iron with protoporphyrin IX and serves as the prosthetic moiety of numerous hemoproteins that are essential for the function of all aerobic cells. Hemoproteins are involved in a remarkable array of crucial biologic functions including oxygen binding (hemoglobin, myoglobin), oxygen metabolism (oxidases, peroxidases, and catalases) and electron transfer (cytochromes). Moreover, heme is the prosthetic group of numerous hemoproteins that synthesize important regulatory or signaling molecules including guanylate cyclase and hydroxylases. Furthermore, heme plays an important role in controlling the expression of numerous proteins (globin, heme biosynthetic enzymes, cytochromes, myeloperoxidase, HO-1, transferrin receptor) and by providing carbon monoxide (CO), which may have a modulatory role akin to that of nitric oxide (NO). Cellular heme levels seem to be tightly controlled; this is achieved by a fine balance between heme biosynthesis and catabolism by the enzyme HO (Voet et al., 1999). Heme requirements vary significantly among various cells and tissues; the most rapid rates of heme biosynthesis occur in erythroid cells and in liver. Even between these 2 tissues there

is a dramatic difference in synthetic rates, because 85% of organismal heme is synthesized in immature erythroid cells whose total number is considerably lower than that of hepatocytes. Hence, on a per-cell basis, the rate of heme synthesis in the developing erythroid cells is at least 1 order of magnitude higher than that in the liver (Voet et al., 1999). Consequently, hemoglobin provides most of the organismal heme for catabolism by HO in macrophages. However, all cells, with the exception of mature erythrocytes and perhaps some other highly differentiated cells, can degrade heme.

Current knowledge regarding intracellular heme trafficking and assembly of hemoproteins is far less than adequate, although some information on the formation of mitochondrial cytochromes has been obtained from experiments with yeast. These studies revealed that heme, which is produced in mitochondria (Figure 1.1), is covalently attached to cytochrome *c* by the enzyme cytochrome *c* heme lyase, localized in the mitochondrial intermembrane space (Voet et al., 1999). Apocytochrome *c* is synthesized in the cytoplasm, transported to the mitochondrial intermembrane space, and subsequently attached to heme by heme lyase. Failure to attach heme to cytochrome *c*, which can be produced by a heme analog, mutation of cytochrome *c*, or by a lack of heme lyase, leads to accumulation of the precursor apocytochrome *c* in the cytoplasm. Additional experiments have suggested that the synthesis of cytochrome *c6* precursor may be coupled to heme availability by a control mechanism operating at the translational level (Voet et al., 1999).

Information on the assembly of many important extramitochondrial hemoproteins (eg, hemoglobin, myoglobin, cytochrome P450) is incomplete, although the role of heme in the processing of a myeloperoxidase is reasonably well understood. Hepatocytes are known to contain a "free" or "uncommitted" heme pool consisting of newly synthesized heme that serves both precursor and regulatory functions (Ponka 1999). In primary cultures of adult rat hepatocytes, 20% of newly formed heme is directly converted to bile pigments, whereas 80% is used for the formation of hemoproteins (Ponka et al., 1973). Hence, it seems that in the hepatocytes, heme is formed in slight excess over its metabolic needs. In erythroid cells, "uncommitted" heme is even more enigmatic and was shown to increase in mitochondria when the cells were treated with protein synthesis inhibitors (Ponka et al., 1973). This suggests that globin chains are probably needed for the release of heme from mitochondria. However, it is also possible that specific heme-binding proteins are involved in the intracellular transport of heme and in its targeting to appropriate intracellular locations. Candidates for these functions include liver fatty acid-binding protein, (Vincent and Müller-Eberhard, 1985) glutathione *S*-transferase, (Harvey and Beutler, 1982) and a heme-binding protein designated HBP23 (Iwahara et al., 1995). HBP23 is a particularly attractive candidate because it binds heme with an affinity that is much higher than that of the other 2 proteins (Iwahara et al., 1995). Interestingly, HBP23 is highly homologous to the mouse macrophage 23-kDa stress protein, which is inducible by oxidant stress in peritoneal macrophages (Ishii et al., 1993; Ishii et al., 1995). These results suggest that HBP23 may also have an antioxidant function. More recently, Taketani and colleagues (1998) purified a novel

heme binding protein with a molecular mass of 22 kDa (termed p22 HBP) and identified its complementary deoxyribonucleic acid (cDNA), the sequence of which revealed that p22 HBP comprises 190 amino acid residues. p22 HBP is ubiquitously expressed in various tissues and is extremely abundant in the liver. It binds heme (*K*d ~ 25 nM), protoporphyrin, and coproporphyrin with relatively high affinities. p22 HBP (both mRNA and protein) is induced during erythroid differentiation, and antisense oligonucleotides to p22 HBP decrease heme biosynthesis in dimethyl sulfoxide (DMSO)-induced murine erythroleukemia (MEL) cells. Hence, p22 HBP may be involved in heme utilization for hemoprotein synthesis and may even be coupled to hemoglobin synthesis during erythroid differentiation (Taketani et al., 1998).

1.1.2 Biosynthesis

1.1.2.1 Overview of Heme Synthesis

Heme biosynthesis involves 8 enzymes, 4 of which are cytoplasmic and 4 of which are mitochondrial (Figure 1.1). The first step occurs in the mitochondria and involves the condensation of succinyl coenzyme A (CoA) and glycine to form Δ-aminolevulinic acid (ALA), catalyzed by ALA synthase (ALA-S). The next 4 biosynthetic steps take place in the cytosol (Voet et al., 1999). ALA dehydratase (ALA-D) converts 2 molecules of ALA to a monopyrrole porphobilinogen (PBG). Two subsequent enzymatic steps convert 4 molecules of PBG into the cyclic tetrapyrrole uroporphyrinogen III, which is then decarboxylated to form coproporphyrinogen III. The final 3 steps of the biosynthetic pathway, including the

insertion of ferrous iron into protoporphyrin IX by ferrochelatase, occur in the mitochondria. Physiological reasons or possible advantages for the mitochondrial localization of the initial and last 3 enzymes, as opposed to the cytoplasmic location of the remaining enzymes, are not known.

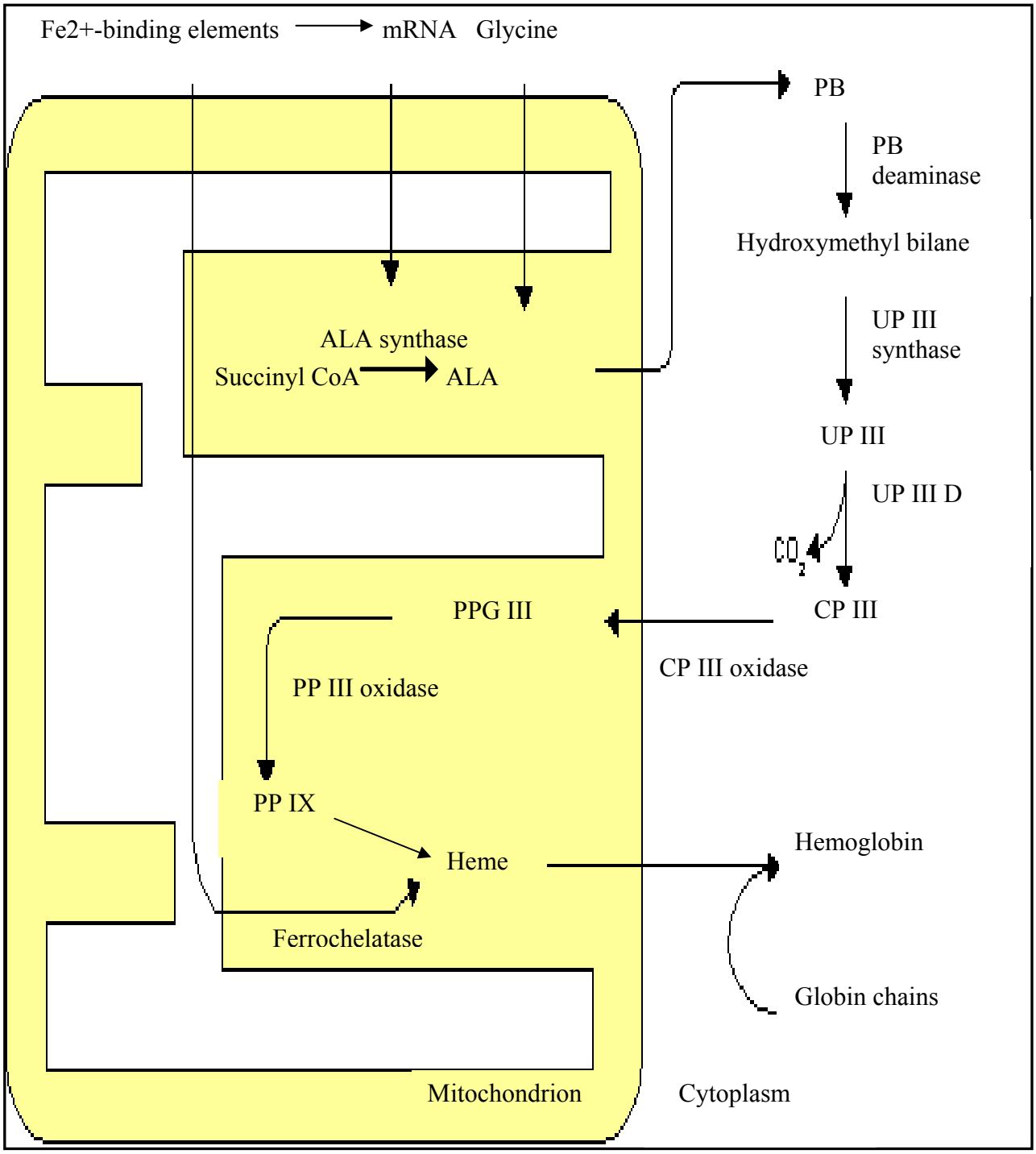

Figure 1.1. The pathway of heme biosynthesis. [Adapted from http://en.wikipedia.org/wiki/Image:Heme_synthesis.png; ALA=d-aminolevulinicacid; PPG=Protoporphyrinogen;PP=Protoporphyrin;CP=Coproporphyrinogen; UP=Uropophyrinogen;UPD=Uroporphyrinogen decarboxylase;PB=Porphobilinogen]

1.1.2.2 Regulation of Heme Biosynthesis

All mammalian heme pathway enzymes have been cloned and the genes encoding these enzymes reside on different chromosomes (Ponka 1999). There are 2 different genes for ALA-S, one of which is expressed ubiquitously and the other is specific to erythroid cells. These 2 genes are responsible for the occurrence of ubiquitous ("housekeeping") and erythroid-specific mRNAs for ALA-S and, consequently, 2 corresponding isoforms of the enzyme. No tissue-specific isozyme is known for ALA-D, but there are subtle differences in 5' UTRs in "housekeeping" and erythroid ALA-D mRNAs. PBG deaminase (Figure 1.1) exists in 2 isoforms; one is present in all cells and the other is expressed only in erythroid cells. However, these isoforms are translated from 2 messenger ribonuceilec acids (mRNAs) that differ solely in their 5' ends. There is no evidence that the ubiquitous and the erythroid enzymes would be different in the rest of the pathway, but variations in mRNAs, caused by the alternative use of the 2 polyadenylation signals, have been reported for coproporphyrinogen oxidase and ferrochelatase (Ponka 1997).

ALA synthase, which condenses glycine with succinate upon decarboxylation, plays an important regulatory role in heme biosynthesis. However, the mechanisms controlling ALA-S expression in the liver, and perhaps in other nonerythroid cells, are dramatically different from those occurring in hemoglobin-synthesizing cells. In the early 1980s, Bishop and colleagues (1981) described significant differences in kinetic and ligand-binding properties between the erythroid and non-erythroid forms of ALA-S of guinea pig, suggesting that there may be tissue-specific isozymes of

ALA-S. Riddle and colleagues (1989) isolated and sequenced ALA-S cDNAs from erythroid cells and livers of chicken and unequivocally showed that 2 separate genes encode the erythroid and the hepatic ALA-S isozymes. The ubiquitous or "housekeeping"*ALA-S* (*ALA-S1* or *ALAS-N*) gene has been assigned to chromosome 3p21 and the erythroid-specific (*ALA-S2* or *ALAS-E*) gene to a distal subregion of Xp11.21.

1.1.2.3 Pathology of Heme Biosynthesis

Disturbances of porphyrin metabolism are known clinically as the porphyrias, which can be caused by defects in any of the heme biosynthetic enzymes. In general, porphyrias are associated with decreased production of heme in erythroid cells. On the other hand, defects in the *ALA-S2* gene lead to hypochromic microcytic anemias caused by decreased heme synthesis in erythroblasts (May and Fitzsimons 1994). The hallmark of this anemia is the ring sideroblast, a pathologic erythroid precursor containing excessive deposits of nonheme nonferritin iron within mitochondria, which shows perinuclear distribution accounting for the ringed appearance. It has been proposed (Ponka 1997) that a combination of 4 factors plays a role in the pathogenesis of mitochondrial iron accumulation in those sideroblastic anemias in which the defect in heme synthesis has been established: (1) iron is specifically targeted toward erythroid mitochondria; (2) this iron cannot be used because of the lack of protoporphyrin IX; (3) there is a lack of heme, the negative regulator of iron uptake; and (4) iron can leave mitochondria only after being inserted into protoporphyrin IX. However, in some sideroblastic anemias, in particular the primary

acquired ones; there is little evidence for inhibited protoporphyrin formation. Recently, a point mutation in mitochondrial DNA coding for one of the subunits of cytochrome *c* oxidase has been demonstrated in hematopoietic cells of a patient with primary acquired sideroblastic anemia (Gattermann et al., 1997). It has been suggested that defective cytochrome *c* oxidase is responsible for the reduced availability of ferrous iron for ferrochelatase, leading to Fe(III) accumulation in mitochondria.

Decreased heme production causing hypochromic microcytic anemias, but without sideroblast formation, can be brought about by a genetic defect in intracellular iron translocation. Fleming and colleagues (1997; 1998) identified a point mutation in the gene encoding Nramp2, a putative endosomal iron transporter that causes decreased iron uptake by erythroid cells of mice with microcytic anemia and of anemic Belgrade rats. There are also genetically based hypochromic microcytic anemias in humans; one attractive possibility is that they are caused by a mutation in Nramp2 gene as well (Hartman and Baker 1996).

1.1.3 Heme Catabolism

Although the major proportion of cellular heme is probably associated with hemoproteins, it is generally assumed that cells contain low levels of heme in an "uncommitted" pool. It is likely that they are equipped with a sensing system to monitor changes in the size of this pool. Recently, a number of diverse proteins known to be regulated by heme were shown to contain a short cysteine-proline-rich

sequence, termed HRM that binds heme (Zhang and Guarente 1995). It should be pointed out that the HRMs are present in almost all eukaryotic species of ALA-S, which suggests their fundamental role in the regulation of this enzyme (Duncan et al., 1999). The HRM is different from sequences found in cytochromes and globins (usually histidine-methionine pair or bis-histidine) that bind heme very tightly, sometimes covalently (cytochromes). It is conceivable that the presence of HRMs on the enzymes involved in heme metabolism represents a mechanism by which "heme sensing" is accomplished (Ponka 1999).

The only physiological mechanism of heme degradation is via HO to biliverdin, CO, and iron (Abraham and Kappas 2005). HO exists as the constitutive isoenzymes HO-1, HO-2, and HO-3 (Cruse and Maines 1988). HO-3 has no activity and is not expressed in humans (Abraham and Kappas 2005). HO-2 is a constitutive isoform (Huang et al., 2001; Scapagnini et al., 2002). HO-1 is an inducible isoform that may be induced through the use of various pharmaceutical agents (Figure 1.2). It was previously shown that overexpression of HO can be successfully achieved through site- and organ-specific gene delivery by means of adenoviral, retroviral, and liposome- based vectors (Abraham et al., 1995; Abraham 2003; Yang et al., 2004).

The heme oxygenase-1 (HO-1) isoform is thought to provide an antioxidant defense mechanism, because it is activated in virtually all cell types, not only by heme but also by hypoxia, inflammatory cytokines, and many types of "oxidative stress." (Glaum and Miller 1993). Poss and Tonegawa (1997) provided direct support for the idea that HO-1 is an important enzymatic antioxidant system. These

investigators generated mice lacking functional HO-1 and demonstrated that, compared with normal fibroblasts, embryonic fibroblasts isolated from HO-1-deficient pups produced high levels of oxygen free radicals in response to treatments by hemin, hydrogen peroxide, paraquat, or cadmium chloride. Moreover, young adult mice deficient in HO-1 were highly vulnerable to mortality and hepatic necrosis when challenged with endotoxin (Poss and Tonegawa 1997). It seems likely that the protective effects of HO-1 are related to this enzyme's capacity to provide biliverdin and bilirubin, both of which are antioxidant agents (Stocker et al., 1987).

Conversely, it can be argued that HO also increases levels of cellular iron that, in its ferrous form, can catalyze the formation of free radicals. Although this may represent a hazard shortly after HO induction, experimental up-regulation of HO-1 by treatment with heme affords protection against subsequent oxidative challenges (Balla et al., 1993). This can be explained by an iron-mediated increase in translation of ferritin, which has antioxidant effects by sequestering iron. In this connection it is pertinent to mention that HO-1 deficient adult mice develop an anemia associated with abnormally low serum iron, indicating that HO-1 is crucial for the recycling of hemoglobin iron and the release of iron from tissue stores (Poss and Tonegawa 1997). Interestingly, Yachie and colleagues (1999) recently identified the first human case of HO-1 deficiency. Mutational analysis revealed abnormalities in both HO-1 alleles. Complete deletion of exon 2 was found in the maternal allele and a 2-nucleotide deletion was present within exon 3 of the paternal allele. The patient, a 6-year-old boy, suffered marked growth retardation and developmental delay associated with

erythrocyte fragmentation, persistent intravascular hemolysis, and marked abnormality of the coagulation/fibrinolysis system. Some of these changes may be related to extremely high serum heme concentration (490 μM, normally not detectable) found in this patient. Nonheme iron deposition was found in renal and hepatic tissues, and a lymphoblastoid cell line derived from the patient was extremely sensitive to hemin-induced cell injury. Hence, numerous phenotypic changes found in this patient, such as growth retardation, tissue iron overload, and vulnerability to oxidative stress, are similar to those found in HO-1 deficient mice (Poss and Tonegawa 1997).

Dennery and colleagues (1998) reported that HO-2 also plays an important role in protection against oxidative injury. These investigators demonstrated that mutant mice lacking HO-2 had substantially increased mortality with chronic hyperoxic exposure. Moreover, they had significantly increased markers of oxidative injury even before hyperoxic exposure. Furthermore, during hyperoxia, lung hemoproteins and iron content were significantly increased without increased ferritin, a situation that suggests an accumulation of redox-active iron. Interestingly, the absence of HO-2 was associated with HO-1 induction, which, however, was not sufficient to protect the mutant mice against hyperoxia-induced oxidative injury (Dennery et al., 1998).

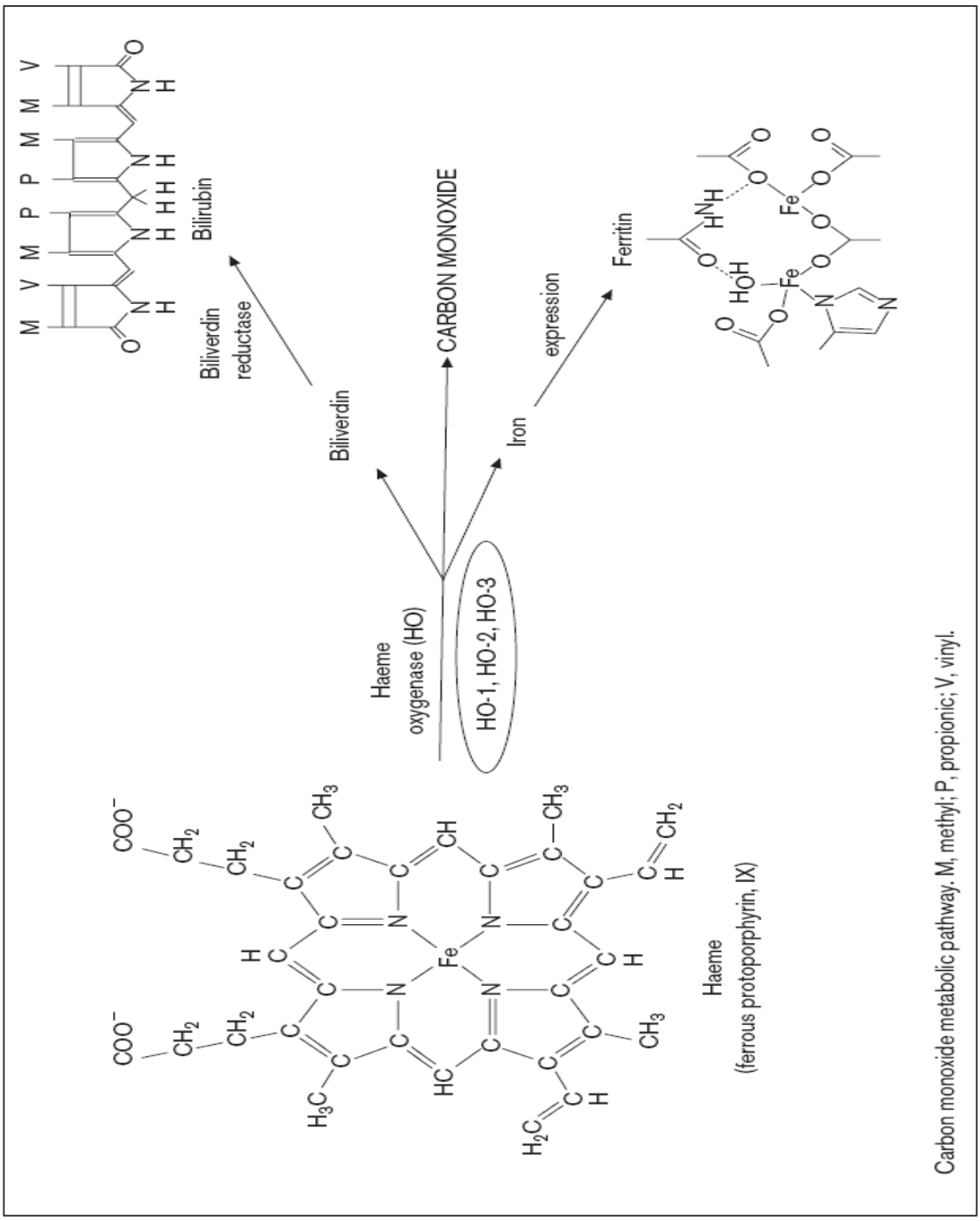

Figure 1.2 HO catalytic breakdown of heme. (adapted from Ndisang et al., Journal of Hypertension 2004, 22:1057–1074).

1.2 The HO/CO system and cardiovascular functions

1.2.1 Introduction

Heme is the prosthetic group of numerous enzymes and is important to cardiovascular homeostasis through regulation of the activity of soluble guanylate cyclase (sGC), NO synthase (NOS), cytochrome P450 (CYP450) monooxygenases, cyclooxygenase (COX), and catalase (Abraham et al., 1996). HO-1 and HO-2 are both viewed as playing a major role in heme breakdown (Dennery et al., 1998) and are alike in terms of mechanism of heme oxidation, cofactor, and substrate specificities, and susceptibility to inhibition by synthetic metalloporphyrins, in which the central iron atom is replaced, for example, by zinc, tin, or other elements (Dennery et al., 1998).

CO is a vasodilator, which has been shown to play an important role in the regulation of basal and constrictor-induced vascular tone in blood vessels (Kaide et al., 2003; Kaide et al., 2004). Upregulation of bilirubin and CO, through the induction of HO-1, have shown promising results in protection against oxidative stress and injury whereas the absence of HO-1, for example, in mice, results in accelerated atherosclerotic lesion formation and vein-graft disease as well as elevated blood pressure (BP), cardiac hypertrophy, and acute renal failure. In addition, the occurrence of organ damage and mortality are more frequent in HO-1 null mice (Perrella and Yet 2003). Induction of HO-1 also prevents cell death, which is attributed to its augmentation of ion efflux and exportation of iron-binding protein

(Ferris et al., 1999). The protective actions of HO-1 or CO are not confined to overtly oxidant processes, but rather extend widely to various pathological processes. Conners and colleagues (1995) were the first to demonstrate that induction of HO-1 has an anti-inflammatory effect. This finding was substantiated by studies from other laboratories (Willis et al., 1996). Furthermore, HO-1 induction has been shown to be cytoprotective in atherosclerosis (Juan et al., 2001), sepsis (Wiesel et al., 2000), diabetes (Pileggi et al., 2001), lung injury (Hashiba et al., 2001), occlusive vascular disease, and ischemia (Guo et al., 2004)

1.2.2 Vascular effects of CO

The vascular production of CO and its vascular effects have been known for decades. Soon after the discovery of the endogenous production of CO in humans during heme catabolism, the vascular effect of CO was detected by Duke and Killick who showed that CO decreased pulmonary vascular resistance (Duke and Killick 1952). Early studies of ductus arteriosus (Coceani et al., 1984), femoral arterial rings (Vedernikov et al., 1989) and rat thoracic aorta (Lin and McGrath 1988) demonstrated the vasorelaxant effects of CO. The endogenous production of CO from the vascular wall, including vascular smooth muscle, endothelium and neurons was later documented.

It is well known that CO can induce relaxation of vascular tissues with different diameters from various species (Zhao and Wang 2002). The affected vascular tissues include aorta, tail artery, pulmonary artery and vein, mesenteric artery, renal arteries,

hepatic arteries, ductus arteriosus and femoral arteries. Exogenously applied CO induced a concentration-dependent relaxation of rat tail artery tissues precontracted with phenylephrine (Wang et al., 1997). The CO-induced vasorelaxation was not due to antagonism of α-adrenoreceptors since CO also relaxed the vascular tissue precontracted with U-46619 (9,11-dideoxy-11α, 9α –epoxymethano-prostaglandin F2α), which induces vasoconstriction mainly by releasing intracellular calcium. The CO-induced vasorelaxation was sustained, but reversible upon the withdrawal of CO, and independent of the presence of an intact endothelium (Wang et al., 1997)**.**

1.2.3 Effects of CO on different cellular components of vascular walls

1.2.3.1 Effect on cultured vascular smooth muscle cells (VSMCs)

Evidence supporting the direct effect of CO on smooth muscle cells (SMCs) has been obtained by studying cultured vascular SMCs in vitro (Ramos et al., 1989). Morita and colleagues (1997) demonstrated that increased endogenous CO production or exposing cells to exogenous CO led to markedly attenuated cell growth. Conversely, inhibiting CO formation or scavenging CO with hemoglobin increased vascular SMC proliferation induced by serum or endothelin stimulation.

1.2.3.2 Role of endothelium in the metabolism and function of CO

1.2.3.2.1 Expression of HO isoforms and production of CO in endothelial cells

The endothelium constitutes a monolayer covering the inner surface of the entire circulatory system. Several vasoconstricting and vasorelaxing factors are

secreted from the endothelium to finely tune the vascular tone (Zhao and Wang 2002). For example, endothelium derived relaxant factor (NO) and hyperpolarizing factor (EDHF) as well as prostacyclin are responsible for the relaxation of vascular smooth muscles, whereas endothelin induces vasoconstriction by acting on endothelin receptors on SMCs (Zhao and Wang 2002).

The vascular endothelia of the intramural vessels of the urethra, bladder, and esophagogastric junction, and the extramural vessels of the bladders of piglets, displayed HO-2 immunoreactivity, but not HO-1 reactivity. HO-2 reactivity was also demonstrated in the endothelial lining of porcine aorta (Sammut et al., 1998). CO generated from endothelial HO-2 is not likely to play an important role in the acute generation of vascular tone since HO-2 is not inducible except in response to adrenal glucocorticoids (Maines et al., 1996).

Interestingly, the acutely regulated release of CO from endothelium was reported in one case. Inhibition of HO activity by tin protoporphyrin IX (SnPPIX) reversed the ACh-induced and NO-independent relaxation of porcine distal pulmonary arteries (Sammut et al., 1998). In the absence of endothelium, ACh failed to relax vascular tissue. Therefore, this study was presented to support the idea that ACh released CO from the endothelium in addition to NO release.

1.2.3.2.2 Mediation of vascular effect of CO by endothelial cells

For CO in the circulation to act on SMCs, it must pass through the endothelium layer of vessel walls. Endothelial cells and their cellular contents may function as

barriers or traps to limit access of CO to SMCs. For example, CO can interact with inducible nitric oxide synthase (iNOS) in the endothelial cells, thus decreasing the number of CO gas molecules diffusing to the SMCs. For CO generated in SMCs or endothelial cells to exert an autocrine or paracrine vasoactive effect, it may stimulate endothelium to modulate the release of endothelium-derived vasoactive factors. Besides its autocrine effect of inhibiting the proliferation of SMCs, the released CO suppresses the production of ET-1 from endothelial cells in a paracrine fashion (Kourembanas et al., 1998).

1.2.4 The vasorelaxant effects of endogenous CO

1.2.4.1 Effect of hemin and other HO inducers on vascular contractility

The vascular effect of endogenous CO has been studied by using the HO substrate and/or inducers such as hemin, heme arginate, or heme-L-lysinate (HLL) (Leffler et al., 1999). Results from *in vivo* studies showed that HO inducers can decrease BP in spontaneously hypertensive rats (SHRs) and increase coronary blood flow in isolated perfused hearts (Levere et al., 1990). Moreover, the development of hypertension in SHRs was chronically retarded by HO inducers such as stannous chloride ($SnCl_2$). Heme-mediated decreases in BP can be prevented by pre-treating the animals with HO inhibitors, suggesting that the vasodilating action of heme is consequent to the formation of HO products (Sacerdoti et al., 1989). These results suggest that endogenous HO products may be responsible for the relaxation of vascular smooth muscle *in vivo*. As biliverdin did not induce vasodilatation and

chelation of free iron had no effect on the HLL-induced vasorelaxation, CO was the only vasoactive molecule resulting from HO induction (Johnson et al., 1999).

After intraperitoneal injection of hemin for 18 h, HO-1 expression in hepatocytes increased in a time-dependent fashion. Concurrently, the vascular resistance in perfused rat liver was reduced by 13% and the CO level in venous effluent was markedly increased (Wakabayashi et al., 1999). An application of oxyhemoglobin that scavenged both NO and CO abolished the hemin treatment-induced reduction of vascular resistance. The specific involvement of CO was further demonstrated by the failure of methemoglobin, which traps NO but not CO, to modulate the hemin effect. Clearly, hepatic circulation is significantly modulated by overproduced CO from extrasinusoidal liver tissues. The vascular effect of CO is also demonstrated in other vascular tissues. For example, the vascular tone of isolated lamb ductus arteriosus was significantly decreased after hemin was added to the bath solution (Coceani et al., 1997).

1.2.5 Mechanisms of CO-induced vasorelaxation

Three major mechanisms have been proposed to explain the vascular effect of CO: 1) the vascular effect of CO may be mostly mediated by sGC pathway; 2) the vasodilatory effect of CO may be related to the modulation of K^+ channel activities (Wang 1998); and 3) inhibition of cytochrome P450 pathway may also be involved in the vascular effect of CO (Coceani et al., 1984) (Figure 1.3). Taking into account the diversity of the responsiveness of different vascular beds from different species to CO, none of these three mechanisms alone can fully explain the vascular effect of

CO. Most likely, all three mechanisms coexist with additional mechanisms, with each one predominant in certain vascular beds (Zhao and Wang 2002).

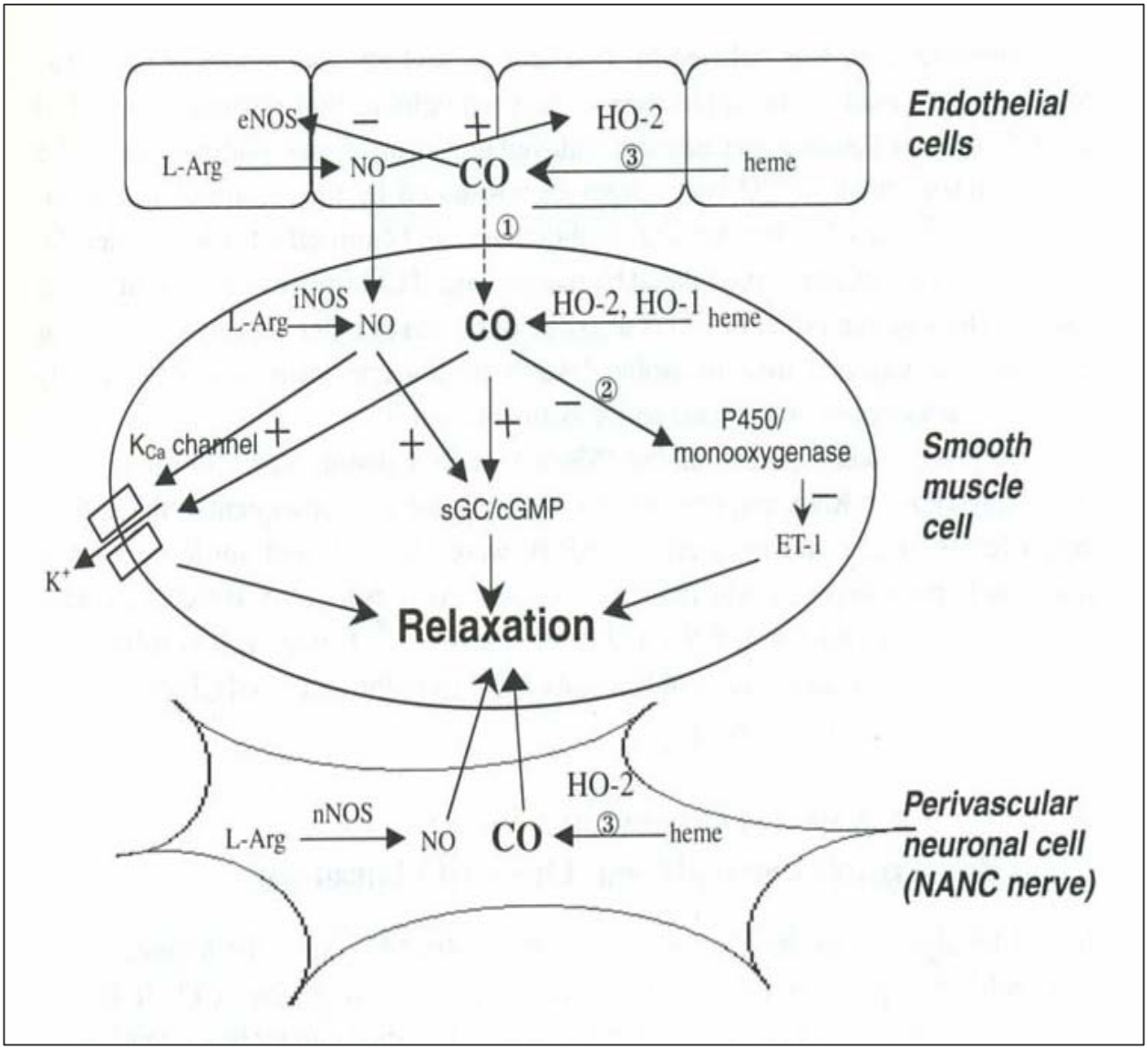

Figure 1.3 Mechanism of CO-induced vascular smooth muscle relaxation. Three major hypotheses for CO-induced SMCs relaxation: cGMP accumulation, large conductance potassium channel activation, and inhibition of the cytochrome P450-monooxygenase system are illustrated. 1- Whether the endothelium-produced CO can diffuse into the underlying SMCs is unclear. 2- Whether this pathway is applicable to adult vascular beds is unclear. 3- The expression and the presence of functional HO-1 are unclear. (Adapted from Zhao and Wang 2002).

1.2.5.1 Stimulation of cGMP pathway

Brune and Ullrich (1987) tested the functional effect of CO on platelets as well as on cyclic nucleotides. Blood obtained from human volunteers was used to prepare platelet rich plasma (PRP). CO bubbled through the PRP for 15 to 30 sec completely inhibited serotonin-induced platelet aggregation. In the presence of a phosphodiesterase inhibitor, isobutylmethyl xanthine (IBMX), CO gassing of PRP induced an increase in cGMP to 143 ± 10% of control values, whereas no change was noted in the content of cyclic adenosine monophosphate (cAMP). These investigators also tested the effect of CO gassing on sGC activity of the 10,000 x g platelet supernatant. In comparison to gassing with nitrogen, CO resulted in an increase in activity of sGC to 402%. These observations are consistent with the view that inhibition of platelet aggregation induced by CO is mediated via an elevation of intracellular cGMP content.

1.2.5.1.1 Effect on cGMP content

Several laboratories have conducted experiments using isolated vascular preparations to examine the effect of CO on mechanical activity and cGMP. For example, Graser and colleagues (1990) examined the relaxation of porcine left descending coronary artery and coronary anterior interventricular vein in response to CO exposure. Rings of these blood vessels were prepared without endothelium for tension measurements during superfusion with a physiological salt solution; the vessels were precontracted by addition of $PGF_{2\alpha}$. Repeated exposure to CO resulted

in a sharp decrease in the tension of the rings: 41.1% for the artery and 67% for the vein. The addition of 10 μM methylene blue, an inhibitor of sGC, resulted in a substantial decrease in the rate and extent of the relaxation induced by CO. This concentration of methylene blue had an effect in the absence of CO as it produced an increase in tension of the arterial and venous rings by 25.1% and 29.9%, respectively. These observations indicate that CO-evoked relaxation might be due to sGC activation (Graser et al., 1990).

Studies conducted by other researchers exploited an sGC inhibitor, H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1one (ODQ) in an attempt to determine the role of the cGMP axis in CO, NO-induced vascular relaxation in rabbit aortic rings (Hussain et al., 1997). In the case of NO, the presence of ODQ at a concentration of 10 μM resulted in substantial blunting of the relaxation response as evidenced by the shift in the dose-response curve. This concentration of ODQ completely eliminated the relaxation response to a dose of CO that induced a 50% relaxation in the absence of the sGC inhibitor. This greater effectiveness of ODQ against CO-induced relaxation compared with relaxation by NO suggests that the CO effect in rabbit aorta is primarily mediated by the sGC/cGMP axis, whereas the NO effect is only partially due to cGMP.

A number of researchers investigated the effects of CO on vascular cells in culture. Morita and colleagues (1995) demonstrated elevation of cGMP content in cultured rat aortic smooth muscle cells after exposure to CO. The peak elevation of cGMP was obtained after 12 h of hypoxic stimulation; this was maintained through

24 h and waned to near-basal values by 48 hr. These observations were discussed in the context of the ability of CO to alter the expression of a number of genes, including those for endothelin-1(ET-1), vascular endothelial growth factor (VEGF), and HO-1, which would, of course, alter the functioning of blood vessels with a duration of action measured in days.

Concurrently, in their studies on rat cell cultures, Christodoulides and colleagues (1995) examined the effects of elevating HO activity on the levels of cGMP in vascular smooth muscle cells and platelets. CO production resulted in a modest elevation in cGMP content of vascular smooth muscle cells in the presence of hemin (an HO inducer). Platelets were studied in co-culture and showed an elevation of cGMP of 23.6% with hemin and 111% with sodium arsenite. These investigators suggested the possibility that HO/CO might function in the circulatory system by acting on platelets as well as on vascular smooth muscles.

In consideration of all these observations, it seems likely that cGMP plays a significant role in mediating the CO-induced relaxation of at least some blood vessels.

1.2.5.1.2 Mechanism of CO activation of sGC

Stone and Marletta (1994) confirmed the ability of CO to interact with and activate sGC purified from bovine lung. CO participated in a six-coordinate complex in which CO and imidazole are comprised at the axial ligands of sGC. In addition, CO induced about a four-fold increase in activity from 221 to 966 nmol

cGMP/min/mg protein. Similar observations were reported by Burstyn and colleagues (1995), who conducted studies on partially purified bovine lung sGC.

In further studies on bovine sGC, Kharitonov and colleagues (1995) found that the dissociation rate constant of the sGC-CO complex was much higher than predicted for a six-coordinate structure. For sGC-CO, they calculated a rate constant much higher than that reported for CO-myoglobin for CO-horseradish peroxidase.

1.2.5.1.3 Activation of sGC by YC-1

The arrival of YC-1 as a pharmacological tool in the study of sGC has given considerable impetus to the consideration of CO as an important regulatory molecule in the vasculature (Zhao and Wang 2002). Friebe and colleagues (1996) reported that YC-1 alone activates bovine lung sGC with maximal activation of about 12-fold at 200 μM and with EC_{50} of 20 μM. Interestingly, they also reported synergism between YC-1 and CO in the activation of sGC. Wegener and colleagues (1997) investigated the actions of YC-1 on rat aortic rings with endothelium present. YC-1 relaxed phenylephrine (PHE)-induced contractions fully at concentrations in excess of 10 μM; this relaxation was enhanced in the presence of zaprinast, a phosphodiesterase inhibitor. These observations are consistent with the idea that YC-1 alone induced relaxation of the aorta by activation of sGC. The mechanism by which YC-1 could enhance the ability of CO to activate sGC may involve alterations to the interaction of CO with the heme of sGC (Zhao and Wang 2002).

1.2.5.2 The effects of CO on K^+ channels on vascular SMCs

Many studies have reported the modulation of ion channels by CO in different cell preparations and proposed different working hypotheses. In most cases, CO acts on calcium-activated potassium channels (K_{Ca}). It was previously shown that CO induced a concentration-dependent relaxation of isolated rat tail artery tissues precontracted with phenylephrine or U46619 (Cao and Wang 2002). The CO-induced vasorelaxation was partially inhibited by blocking either the cGMP pathway or BK_{Ca} channels. When both the cGMP pathway and K_{Ca} were blocked, the CO-induced vasorelaxation was completely abolished (Wang et al., 1997). These results suggest that CO may activate both a cGMP signaling pathway and K_{Ca} channels in isolated rat tail artery SMCs.

1.2.5.3 The effect of CO on cytochrome P450

Cytochrome P450-linked mono-oxygenase is responsible for the generation of vasoconstricting substances, such as an arachidonic metabolite or ET-1 (Harder et al., 1996). A decreased formation of these vasoconstrictors would lead to vascular relaxation (Wang 1998). CO and cytochrome P450 function are closely related. CO is an inhibitor of cytochrome P450 and the levels of cytochrome P450 are controlled by the availability of cellular heme which can be catalyzed to CO and biliverdin (Levere et al., 1990). Therefore, it has been hypothesized that the vascular effect of CO may result from the inhibition of a cytochrome P450-dependent mono-oxygenase.

In the lamb ductus arteriosus, exogenously applied CO and most of the known cytochrome P450 inhibitors relaxed the tissue precontracted with either indomethacin or potassium chloride (KCl). The CO-induced relaxation was maximally reversed by light illumination at 450 nm, indicating the involvement of cytochrome P450 in the process (Coceani et al., 1988).

Conversely, Wang and colleagues reported that the CO-induced relaxation of tail artery tissues from adult rats was not affected by the presence or absence of 4-phenylimidazole (4-PD), an antagonist of cytochrome P450 (Wang et al., 1997; Wang 1998). Indeed, 4-PD alone relaxed the precontracted endothelium-free rat tail artery. After the 4-PD-alone relaxation reached the maximum, however, additional relaxation could be induced using CO. The CO-relaxed vascular tissues could also be further relaxed by 4-PD. These results, together with the observation that the simultaneous inhibition of both cGMP pathway and K_{ca} channels completely eliminated the CO-induced relaxation, do not support the involvement of the cytochrome P450 system in the CO-induced relaxation of rat tail artery tissues.

1.2.6 The HO/ CO system and NO

1.2.6.1 Introduction

CO, like NO, binds to iron in the heme moiety of heme proteins. The two gasotransmitters share many common downstream signaling pathways and have the same regulatory functions. On the other hand, there are some functional differences between CO and NO. For example, NO mainly mediates glutamate effects at N-

methyl-d-aspartate (NMDA) receptors, whereas CO is primarily responsible for glutamate action at metabotrophic receptors. As another example, CO becomes a stimulatory modulator of sGC when the tissue level of NO is low. Conversely, CO inhibits sGC activity with a high tissue NO level (Kajimura et al., 2003). The potential interplay between CO and NO also occurs at different levels, being synergistic or antagonistic, which provides an integrated mechanism for the fine-tuning of cellular functions. HO resembles NOS in that the electrons for CO synthesis are donated by cytochrome P450 reductase, which is 60% homologous at the amino acid level to the carboxyl terminal half of NOS (Foresti and Motterlini, 1999).

The induction of HO-1 appears to be a fundamental defensive system to counteract cellular and tissue damage and the importance of this protein in the maintenance of vascular function is best exemplified by the first case of human HO-1 deficiency described in the literature (Yachie et al., 1999). NO synthase and HO-2 are co-localized in vascular endothelial cells and in some enteric neurons. NO and CO appear to act as co-neurotransmitters in the enteric nervous system (Xue et al., 2000). Smooth muscle cells were depolarized in both HO-2 and neuronal NO synthase null mice. The double null mice (HO-2 and neuronal NO synthase) manifested additional depolarization, reflecting additive effects of the two enzymes (Xue et al., 2000). Electrical field stimulation of jejunal smooth muscle strips from HO-2 null mice hardly produced any response despite expression of NO synthase. However, application of exogenous CO restored normal relaxation, indicating that the NO system does not function in the absence of CO generation. It was thus concluded

that NO and HO-2-derived CO act similarly to control vascular tone under physiological conditions (Xue et al., 2000) (Figure 1.4).

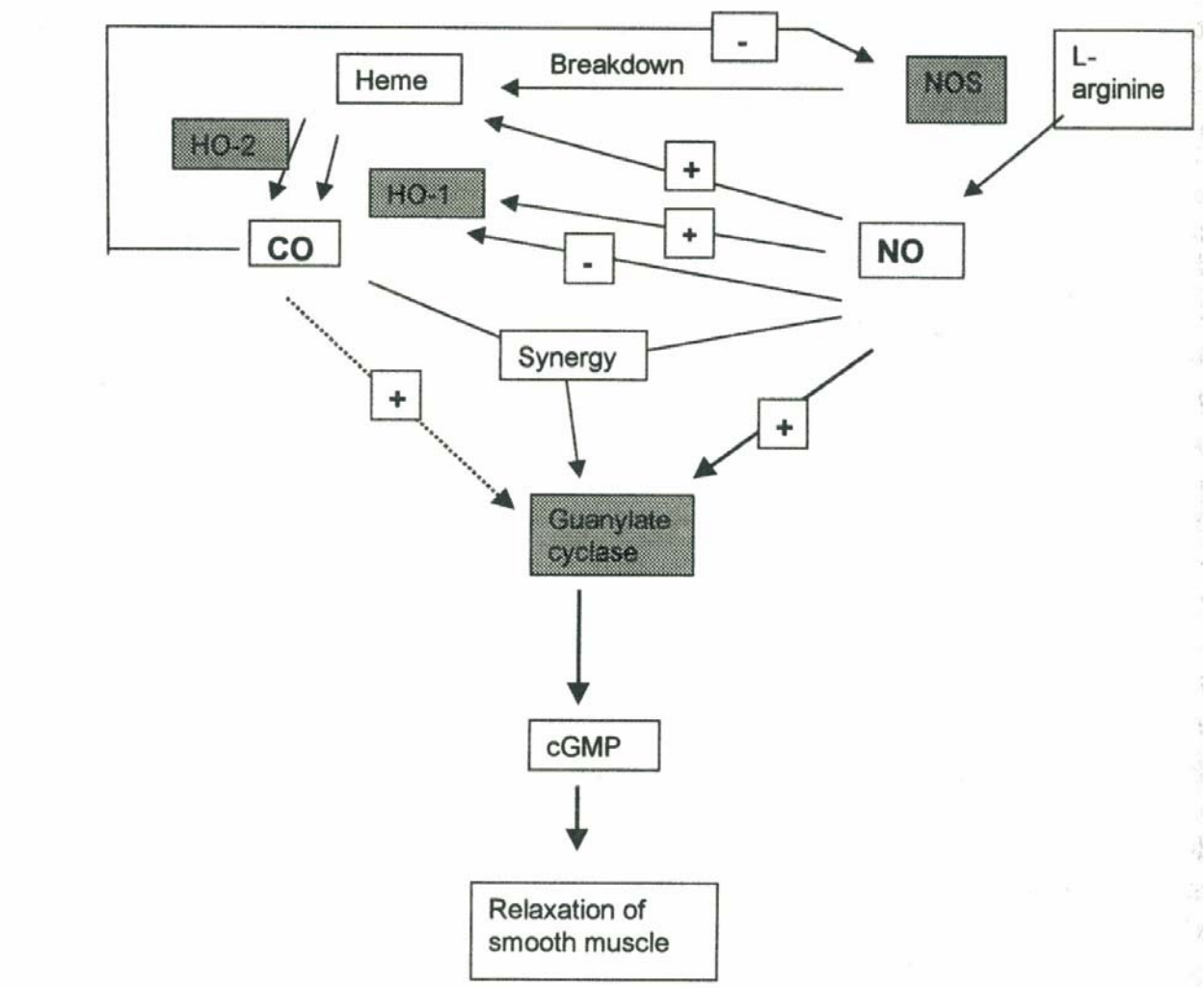

Figure 1.4 Summary of the interactions between CO and NO systems. (Adapted from Carson et al., 2004)

1.2.6.2 Effects of NO on HOs

Several studies reported that in many different tissues exposure to NO or NO donors results in increased HO activity, suggesting that this may be a generalized response elicited by NO (Durante et al., 1997; Motterlini et al., 1996; Clark et al., 1997).

The effect of NO on heme catabolism may also be independent of the interaction of NO with HO enzymes. In fact, NO can complex with heme and the formation of nitrosyl-heme appears to inhibit heme degradation by HO. Juckett and colleagues (1998) showed that incubation of HO-1 rich microsomes with NO donors causes a concentration-dependent inhibition of HO activity. The authors hypothesized that this might be a transient event and, as the concentration of NO declines, the nitrosyl-hemes will dissociate and free heme would be available as substrates for HO inducers.

1.2.6.3 Effects of CO on NO synthases

CO can cause a release of NO from its intracellular pool (Thorup et al., 1999). In renal resistance arteries, 100 nM CO resulted in the release of NO. Consecutive applications of brief pulses of CO attenuated the amplitudes of the consecutive releases of NO (Thorup et al., 1999). Intracellular redistribution of NO is one hypothesis for this CO-mediated NO release (Wu and Wang 2005). Different equilibrium constants for metallic binding of the two gasotransmitters are the basis of this hypothesis. As is well known, NO is the most reactive physiological gas,

comparable to O_2 in its effective size and polarity. NO binds to Fe(II)-heme protein as soon as it enters the binding pocket (Scott et al., 2001). NO has a much greater overall association constant with Fe(II)-heme protein than that of CO. The affinity of NO for Fe(II) of hemoglobin, for example, is 1500 times greater than that of CO (Keilin, 1966). On the other hand, CO has a significantly longer dissociation constant than that of NO or O_2 (Gibson et al., 1986). This property facilitates CO-induced NO release from Fe(II)-heme proteins (Moore and Gibson, 1976). The presence of NO sinks, such as reduced thiols, will facilitate the displacement of NO from Fe(II) by CO. However, these rationales are based on chemical reactions in aqueous solutions *in vitro*. Whether or not the CO-induced NO release/displacement in living cells can be fast enough and efficient enough to have a physiological meaning has not been fully addressed (Wu and Wang 2005).

CO can directly bind to and inactivate NOS, decreasing the enzyme activity in some preparations (Ding et al., 1999). This may explain the arteriolar NO dysfunction induced by the overproduction of CO, leading to the development of salt-induced hypertension (Johnson et al., 2003). Endogenous CO has been suggested to control constitutive NOS activity (Turcanu et al., 1998). Inhibition of purified macrophage NOS (iNOS), purified cerebellar nNOS, and endothelial NOS (eNOS) by CO has been reported (Thorup et al., 1999). There is no report to date that CO can directly activate NOS (Wu and Wang 2005).

1.3 The HO/CO system and hypertension

1.3.1 Introduction

CO is commonly recognized as an environmental toxin that arises from the incomplete combustion of fossil fuels (Penney 1990). However, the biological research community now recognizes that living systems also generate CO endogenously via heme metabolism in a reaction catalyzed by HO. Endogenously formed CO is now gaining acceptance as a potentially important regulator of cardiovascular functions (Johnson et al., 1999).

In 1991, Marks and colleagues published a theoretical work noting the ability of CO to activate sGC to increase the formation of cGMP. Since cGMP was established as a second messenger that promotes relaxation of vascular smooth muscle, Marks et al suggested that endogenously formed CO might stimulate the formation of cGMP to promote vasodilation. Reports regarding regulation of HO expression accumulated, but only the most recent studies, those demonstrating that endogenous CO can be acutely manipulated to affect BP, stimulated speculation that endogenous CO may be involved in the development of hypertension (Johnson et al., 1999).

1.3.2 Hypertension: A failure of BP regulation

BP is regulated in a manner that allows for sufficient perfusion of tissues while minimizing the risk for pressure-related organ damage. When arterial pressure rises to

levels that significantly increase the risk of such damage, the condition is known as hypertension (Johnson and Johnson 2002).

Current evidence suggests that the heme-HO-CO system may interact with BP in two ways. First, it appears that the heme-HO-CO system may impact vascular tone, cardiac output, and water and electrolyte homeostasis to affect BP (Johnson et al., 1999). On the other hand, it seems that disorders of BP regulation can lead to metabolic alterations that may have an impact on the heme-HO-CO cascade. Alterations of the heme-HO-CO cascade may be considered causes and/or results of hypertension (Ishizaka et al., 1997).

1.3.3 HO/CO system and regulation of BP

In awake normotensive rat models, intrapertioneal administration of zinc deuteroporphyrin 2,4 bis-glycol, a known HO inhibitor, systemically inhibits CO production and produces a rapid rise in arterial pressure (Johnson et al., 1995). This systemic blockade of HO activity that increases arterial pressure is accompanied by a decrease in cardiac output and paralleled by an increase in calculated total peripheral resistance. In addition, other HO inhibitors have been shown to increase BP and exacerbate angiotensin-II-induced hypertension (Johnson et al., 1995).

In the SHR, a model of genetically-based hypertension, HLL, a highly-soluble heme preparation, has been shown to produce a fall in BP within minutes. HLL-induced decreases in BP can be blocked by pre-treatment with an inhibitor of HO. Intraperitoneal administration of CO has been shown to exert a vasodepressive action

in this rat strain, while the same dose exerts little or no effect in normotensive rats. In contrast, neither the iron nor the biliverdin product can be related to the fall in BP. It is thus conceivable that endogenously formed CO is involved in the regulation of BP (Johnson and Johnson 2002).

1.3.4 CO and neural regulation of BP

The central nervous system plays a vital role in CO-mediated regulation of BP. Inhibitors of HO that cross the blood-brain barrier, such as zinc deuteroporphyrin 2,4 bis-glycol, cause rapid sustained increases in arterial pressures (Johnson et al., 1997). In contrast, HO inhibitors that can cause systemic inhibition of CO production but are unable to pass the blood –brain barrier appear to have little effect on BP. The actions of CO within the CNS, more specifically within the nucleus of the tractus solitarius, are likely to play a very important role in regulation of BP (Johnson and Johnson 2000).

It appears that the central action of CO is to suppress the sympathetic activity, and that its regulatory actions likely arise both in chemoreceptors and in integrating tracts of the nucleus of the tractus solitarius (Johnson and Johnson 2002). It also appears that the development of hypertension may potentially arise from aberrations in endogenous CO formation in the nucleus of the tractus solitarius (Johnson and Johnson 2002).

1.3.5 Endogenously-formed CO and the regulation of vascular tone

The toxicology literature established CO as an activator of sGC and an agent that relaxes vascular smooth muscle (Karlsson et al., 1985). CO production in vascular smooth muscle can be accelerated in response to heme and inhibited upon exposure to certain metalloporphyrins such as zinc deuteroporphyrin 2,4 bis-glycol or chromium mesoporphyrin (Cook et al., 1995). Heme-induced dilation can be prevented by inhibitors of HO. These features suggest that heme-induced dilation of vascular smooth muscle is the result of the generation of an HO product (Kozma et al., 1997).

The vasoconstrictive effects of HO inhibitors seem to arise specifically from the inhibition of CO synthesis as they can be blocked by authentic CO, but not by other vasodilators such as sodium nitroprusside (Kozma et al., 1999). The specific mechanism underlying CO-induced vasorelaxation is unclear. CO is an activator of sGC, but CO-induced dilation can occur independently of sGC activity (Kozma et al., 1999). Considerable evidence suggests CO may confer BP effects through interactions with potassium channels, interference with P-450-mediated functions, or by modulating endothelin formation (Coceani 2000). Furthermore, the typical levels of HO-mediated formation of CO may be either increased or decreased to promote vasodilation or vasoconstriction, respectively. This suggests that endogenously formed CO qualifies as a physiological regulator of vascular tone (Johnson and Johnson 2002).

1.3.6 CO and renal function during hypertension

Hypertension is associated with abnormal renal functions. Normally, increases in renal perfusion pressure promote renal sodium/water excretion to decrease circulating blood volume and suppress the vasoconstrictive- and sodium/water-retaining influences of the renin-angiotensin-aldosterone system (Hall et al., 1996). Renal levels of HO-2 mRNA are significantly increased in stroke-prone SHRs as compared with Wistar Kyoto rats (Seki et al., 1997). This suggests that endogenously-formed CO may modify renal function in some genetically transmittable forms of hypertension. Administration of heme substrate lowers BP in SHRs, and increases renal HO mRNA and HO activity (Johnson and Johnson 2002).

It has been suggested that renal medullary blood flow is important to the long-term control of arterial BP (Cowley 1997). The levels of HO-1 and HO-2 in the renal medulla are higher than those expressed in the renal cortex (Zou et al., 2000). Interstitial infusion of an inhibitor of HO-mediated formation of CO decreases renal medullary blood flow and lowers local concentration of cGMP. Such findings are initial evidence that the HO-CO system may serve as an antihypertensive by maintaining the constancy of renal medullary blood flow (Johnson and Johnson 2002).

HO-1 has been suggested to regulate renal excretory functions in angiotensin-II-dependent hypertension (Aizawa et al., 2000). In kidneys taken from normotensive rats, HO-1 was expressed primarily in the basal sides of renal tubules. Angiotensin-II infusion shifts HO-1 expression to the tubular epithelial cells. In rats made chronically hypertensive by angiotensin-II infusions, administration of hemin protects

against angiotensin-II-induced decreases in glomerular filtration rates and decreases urinary protein excretion. In contrast, administration of the HO inhibitor, zinc protoporphyrin, further aggravates angiotensin-induced decreases in glomerular filtration rates and exacerbates proteinuria (Aizawa et al., 2000).

The renin-angiotensin-aldosterone system is of paramount importance in hypertension. Decreases in renal perfusion pressure and increases in renal sympathetic nerve activity stimulate the renal release of renin (Hall et al., 1996). Renin, in turn, drives the formation of angiotensin-II and aldosterone which promote vasoconstriction and sodium/water retention, respectively.

CO has been shown to increase plasma renin activity, but it also inhibits the formation and release of aldosterone (Penney 1990). If these properties are also displayed under physiological conditions, then endogenously formed CO could potentially promote vasoconstriction by driving the formation of angiotensin-II, while simultaneously interfering with sodium/water retention consequent to formation of aldosterone (Johnson and Johnson 2002). Conversely, suppression of the HO-CO system may promote hyperaldosteronism in the face of relatively normal levels of renin and angiotensin. The pathological and physiological consequences of such uncoupling of aldosterone formation from renin are unclear, but it is speculated that endogenously formed CO might be important in establishing aldosterone release in response to angiotensin formation (Johnson and Johnson 2002).

1.4 The HO/CO system and erectile dysfunction (ED)

1.4.1 Pathophysiology of ED

Normal erectile function requires the involvement and coordination of multiple regulatory systems and is thus subject to the influence of psychological, hormonal, neurological, vascular and cavernosal factors. An alteration in any of these factors may be sufficient to cause ED, but in many cases a combination of several factors is involved (Moreland et al., 2001). Alterations in blood flow to and from the penis are thought to be the most frequent causes of ED. Penile arterial vasodilation and relaxation of the trabecular smooth muscle initiate erection. This allows filling of the sinusoids and entrapment of pressurized blood in the corpora cavernosa. These are the primary hemodynamic events that initiate and maintain penile erection. An increase in intracavernous pressure of 50-90 mm Hg, depending on the geometry and penile tissue factors, is required for erection with sufficient rigidity for vaginal intromission (Moreland et al., 2001).

Proximal arterial stenosis and an increase in penile vascular arterial resistance can lead to organic ED. A higher prevalence of ED is observed in patients with cardiovascular risk factors, such as hyperlipidemia, hypertension, smoking and diabetes (Kloner 2005).

The prevalence of ED is increased with the accumulation of cardiovascular risk factors, as occurs in cardiovascular disease. Indeed, ED can be a symptom of vascular

disease (Kloner 2005). The association of impotence with vascular disease is well-documented in the literature (Billups 2005; Thomas et al., 2005; Carson and Lue 2005). Prolonged illness has been thought to account for a great portion of the decline in sexual function with aging.

Alterations in corpus cavernosal arterial inflow (CAI) and corporal veno-occlusive dysfunction (CVOD) are thought to be the two most frequent causes of organic ED (Vlachopoulos et al., 2005). Both decreased CAI and CVOD have been reported in patients with hypertension, myocardial infarction, cerebrovascular accidents, peripheral vascular disease and following coronary arterial bypass surgery (Carson and Lue 2005).

Wabrek and Burchell (1990) reported ED in 64% of 131 males aged 31-86 years hospitalized for acute myocardial infarction. Several studies have correlated a prevalence of ED with an increasing number of vascular risk factors (Nehra et al., 1995). Shabsigh and colleagues (2005) reported that smoking, diabetes, and hypertension are risk factors for vasculogenic ED, and abnormal penile vascular findings increased significantly as the number of risk factors increased with ED. In another study, Virag and colleagues, 1985, investigated the distribution of four main arterial risk factors of diabetes, cigarette smoking, hypertension, and hyperlipidemia among patients with ED. The study reported that hypertension, smoking, diabetes, and hyperlipidemia were all significantly more common in this cohort of patients than in the general population.

1.4.2. ED in hypertension

The relationship between hypertension and erectile function, often complicated by the effects of antihypertensive medications, has not been established definitively (Feldman et al., 2000). Possible etiologies for ED secondary to hypertension include vascular damage due to hypertensive changes as well as hormonal abnormalities such as elevated prolactin levels (Jaffe et al., 1996). A study of 24 patients with ED and untreated essential hypertension showed decreased levels of free and total serum testosterone compared with normal controls (Hughes et al., 1989). These hormonal findings were supported by a study of 1132 men aged 30 to 79 years that found an inverse relationship between BP and serum testosterone levels (Khaw and Barrett-Connor, 1988).

The Massachussets Male Aging Study (MMAS) 9-year follow-up study found that hypertension was an independent, although modest, predictor of ED (Feldman et al., 2000). Sleep studies in 175 patients with hypertension and erectile problems showed significantly lower penile rigidity measured by strain gauge plethysmography compared with 110 normotensive male controls with similar subjective erectile problems (Hirshkowitz et al., 1989). A study of 1128 patients aged 16 to 80 years found only a "meagre" relationship between hypertension and ED (Newman and Marcus 1985). After controlling for diabetes mellitus, tobacco use and hyperlipidemia, hypertension was not found to be an independent predictor of vasculogenic ED in 440 impotent men (Virag et al., 1985). Another study evaluated 32 hypertensive men with ED and 78 normotensive men with ED with regard to age,

body mass index, hormonal profile, penile arterial flow, risk factors for arterial disease, psychiatric disease, and neurologic disease measured by pudendal nerve conduction studies (Jaffe et al., 1996). This study found that, although hypertensive patients had more coronary artery disease, no direct evidence supported an association between hypertension and arteriogenic impotence, as measured by the peak systolic velocity and resistivity index, in patients with mild to moderate hypertension.

Almost every class of antihypertensive medication has been implicated in causing ED; however, most of these studies, published as case reports or patient surveys, have been relatively subjective and uncontrolled (Wein and van Arsdalen 1988). To implicate a medication as a cause of ED, a study must show a reproducible dose-related effect that stops after the medication is discontinued (Wein and van Arsdalen 1988). Unfortunately, only few such studies exist.

The mechanisms of action by which antihypertensive medications cause ED are currently unknown. Some investigators have theorized that antihypertensive medications affect erectile function by decreasing BP, which reduces the perfusion pressure needed to maintain sufficient blood flow for erections through atherosclerotic penile arteries (Benet and Melman 1995). However, other studies have noted that, when BP levels are monitored after initiation of antihypertensive therapy, changes in BP level are not correlated with sexual function (Rosen et al., 1994). Also, if lower BP level was the primary etiology of ED, all classes of antihypertensive agents should be expected to have relatively similar effects on erectile function

because of their efficacy in lowering pressure, which has not been seen (Kloner 2005). Other investigators have suggested these medications may exert a hormonal effect. β-blockers have been associated with decreased free and total testosterone levels in placebo-controlled trials (Kloner 2005).

β-blockers often have been cited as one of the medications most frequently associated with onset of ED (Kloner 2005). Different classes of β-blockers have been postulated to have differential effects on erectile function, with the nonselective β -blockers (eg, propranolol) having more deleterious effects than the more cardioselective medications (eg. atenolol, metoprolol) (Weiss 1991). A dose-related phenomenon with propranolol use was suggested by another study, which showed that patients receiving propranolol dosages exceeding 120 mg/d developed ED at a higher rate than patients who received lower dosages of the same medication (Warren and Warren 1977). In comparison, a randomized placebo-controlled trial of 63 patients given either placebo or 95 mg/d of sustained-release metoprolol for 4 months after percutaneous coronary angioplasty found no significant difference in sexual function between the 2 groups (Franzen et al., 2001).

Centrally acting antihypertensive agents such as clonidine and methyldopa also affect sexual function (Kloner 2005). These medications cause intracavenosal pressure changes in animal models, and human studies have noted deleterious effects on erectile function, decreased libido, and ejaculatory problems (Weiss 1991). Postulated mechanisms of effect on sexual function with these centrally acting

medications have included increased prolactin levels and a direct effect on α-adrenergic receptors in the central nervous system (Mroczek et al., 1972).

Diuretics, especially thiazides have been identified traditionally as common causes of new-onset ED (Bansal 1988). However, there has been disagreement regarding the effects of diuretics on erectile function. Many studies found that only rarely have these medications been implicated convincingly as the cause of patient ED (Wein and van Arsdalen 1988). An exception is spironolactone, which exerts an antiandrogen effect and can cause gynecomastia and ED in a small percentage of patients (Horowitz and Globe 1979).

α-blocking agents have been implicated as a cause of ED in some studies, whereas other studies have reported minimal effects on erectile function (Meinhardt et al., 1997). However, α-blockers are a well-known cause of retrograde ejaculation secondary to a reversible relaxation of bladder neck smooth muscle (DeBusk et al., 2000). The 2 classes of medications least associated with ED are calcium channel blockers and angiotensin-converting enzyme inhibitors (King et al., 1983). Although some case studies have reported a relationship between calcium channel blockers and ED, most studies suggest that this effect is minimal and that any relationship is likely secondary to a decrease in BP with consequent reflex sympathetic activation (Meinhardt et al., 1997). Angiotensin-converting enzyme inhibitors also have low rates of secondary ED associated with their use in both animal and human studies (Meinhardt et al., 1997).

1.4.3 The role of HO/CO system in mediation of ED in SHR

The basal tone of the corpus cavernosum smooth muscle is controlled by complex events coordinated at the level of the central and peripheral nervous system. The sympathetic nervous system ensures flaccidity by producing an α-adrenergic-dependent tone of the corporal smooth muscle maintaining the penis in a flaccid state, thus minimizing intracavernosal pressure (ICP) and blood flow (Giuliano et al., 1997). On sexual stimulation, penile erection, occurring in response to the activation of pro-erectile autonomic pathways, is greatly dependent on adequate inflow of blood to the erectile tissue and requires coordinated arterial endothelium-dependent vasodilatation and sinusoidal endothelium-dependent corporal smooth muscle relaxation (Burchardt et al., 2001).

A major determinant of erectile capacity and function is a rise in ICP, being an important predictor of penile rigidity and erectile function (Giuliano et al., 1993; Udelson et al., 1998). Electrical stimulation of the cavernosal nerves of normal rats elicits a 60-90 mm Hg increase in ICP, which is similar to ICP in humans during full erection stage (Udelson et al., 1998). Previous reports demonstrated that the electrical stimulation-evoked plateau phase ICP was severely reduced in adult SHR compared to age-matched normotensive WKY and SD rats (Behr-Roussel et al., 2003).

NO is the principal peripheral pro-erectile gasotransmitter that is released by both nonadrenergic, noncholinergic neurons and the sinusoidal endothelium to relax corporal smooth muscle through the cGMP pathway (Chamiot-Clerc et al., 2001; Giuliano and Rampin, 2000) resulting ultimately in increased ICP (Behr-Roussel et

al., 2003). This increase in ICP activates pressure-dependent veno-occlusive mechanisms to limit the outflow of blood, thus further promoting elevated ICP and erectile response (Behr-Roussel et al., 2003).

Previous studies have shown that HO-2 protein is localized in the major pelvic ganglion and the nerves distributing to the penis, urethra, bladder neck, vas deferens and prostate in mice (Burnett et al., 1998). Hedlund and colleagues (2000) detected immunoreactivity for HO-1 and HO-2 enzymes in nerve structures and endothelium of human corpus cavernosum and corpus spongiosum. CO, produced by HO is believed to be one of the major transmitters in these pelvic ganglions and nerves. Furthermore, Ushiyama and colleagues (2004) demonstrated that endogenous CO has an important role in development of ED in SHR. Levels of CO-dependent relaxation of the cavernous tissue were suppressed in adult SHR as compared with WKY. In both rat species CO-dependent relaxation of the cavernous tissue was blocked by HO inhibitors. Increased levels of HO gene expression in organs such as the aorta, left ventricle, and kidney in SHR, which have impaired NO system, suggests that the HO/CO system might take over the role of the NO system when the latter is impaired (Ushiyama et al., 2002). However, in regard to hypertensive ED, crosstalk between the neurogenic NO and CO systems may not be compensatory (Ushiyama et al., 2004). Such a difference may result from the different frequency-dependence for the release of CO or NO from nerves (Ushiyama et al., 2004).

2. Rationales, hypotheses and objectives

2.1 Rationales

1. Hemin and other heme derivatives, HLL and heme-L-arginate, have been shown to markedly decrease high BP in SHR, but not in normotensive Wistar-Kyoto (WKY) rats (Martasek et al., 1991; Ndisang et al., 2002). Not all metabolic effects of heme are beneficial. Heme is hydrophobic, readily entering cell membranes. This will greatly increase cellular susceptibility to oxidant-mediated killing (Balla et al., 1991). Heme also acts as a catalyst for the oxidation of low-density lipoprotein, generating products toxic to endothelium (Camejo et al., 1998). The toxic effects of heme may manifest themselves in a number of pathologies. These include not only acute conditions such as intravascular hemolysis, leading to renal failure, but also more insidious processes such as atherogenesis in which intralesional deposits of iron have been observed (Hunter et al., 1991). Free hemoglobin (Hb) in plasma, when oxidized, can provide heme to endothelium, which greatly enhances cellular susceptibility to oxidant-mediated cell injury (Balla et al., 1995). In these situations, the expression and functions of HO are critical in metabolizing heme to counteract its detrimental effects. The central importance of HO-1 was recently highlighted by discovery of a child with HO-1 deficiency, who exhibited extensive endothelial damage (Yachie et al., 1999). Similar endothelial damage as well as hepatic and renal cytotoxicities have been observed in transgenic mice deficient in HO-1 (Poss and Tonegawa 1997). In

both the human patient and mice lacking HO-1, very high concentrations of circulating heme were observed. **These recent reports necessitate development of a means to monitor circulatory heme levels in different pathological situations and in using hemin therapy to lower high BP. While the methodology to measure serum heme levels has been established long ago (De Duve 1948), metabolism of the injected hemin or other heme derivatives when used to lower BP has not been investigated.**

2. Hemin, a powerful inducer of HO-1 (Levere et al., 1990) has long been successfully used to treat porphyria. A common side-effect of this drug therapy is the occurrence of low BP (Lamon et al 1979). Interestingly, previous reports showed that the administration of HO-inducers like hemin or $SnCl_2$ for 4 consecutive days restored BP to physiological level in young (8 weeks) SHR but not in adult SHR (Ndisang et al., 2004). These studies ascribed the hypotensive effect of hemin therapy to the upregulated expression of HO-1. Consequentially, increased CO production resulted in relaxation of VSMC through the elevation of cGMP levels and/or stimulation of K_{Ca} channels (Morita et al., 1995; Ndisang et al., 2002). BP in young SHR is continuously increasing, while adult SHR have an established hypertension (Escalante et al., 1991). The reason for this age-dependent effectiveness of HO-1 inducers on the regulation of BP remains unclear. Gutsmann-Conrad et al (1999) suggested that since the transcription and translation of some proteins may be slower in adults the activity/expression of important targeted proteins unaffected by acute treatment may be increased by chronic therapy. **There is no published report in**

literature examining the effect of prolonged hemin treatment (beyond 4-7 days) on the BP of adult SHR. Furthermore, preliminary unpublished observations from our lab showed that prolonged hemin administration can lead to a significant decrease in BP of adult SHR (Wang et al., 2005). Following this logic, it can be perceived that hemin for sufficient duration of time may normalize the BP and have long-lasting effect in SHR with established hypertension.

3. A major determinant of erectile capacity and function is a rise in ICP, being an important predictor of penile rigidity and erectile function (Giuliano et al., 1997; Burchardt et al., 2000). Electrical stimulation of the cavernosal nerves of normal rats elicits a 60-90 mmHg increase in ICP, which is similar to ICP in humans during full erection stage (Uldeson et al., 1998). Previous reports demonstrated that the electrical stimulation-evoked plateau phase ICP was severely reduced in adult SHR compared to age-matched normotensive Wistar-Kyoto (WKY) and Sprague-Dawley (SD) rats (Behr-Roussel et al., 2003). NO is the principal peripheral pro-erectile gasotransmitter that is released by both nonadrenergic, noncholinergic neurons and the sinusoidal endothelium to relax corporeal smooth muscle through the cGMP pathway (Chamiot-Clerc et al., 2001; Giuliano and Rampin 2000), resulting ultimately in increased ICP (Behr-Roussel et al., 2003). This increase in ICP activates pressure-dependent veno-occlusive mechanisms to limit the outflow of blood, thus further promoting elevated ICP and erectile response (Behr-Roussel et al., 2003).

CO produced by HO is believed to be one of the major transmitters in pelvic ganglions and nerves (Ushiyama et al., 2004). Ushiyama et al., 2004, demonstrated that endogenous CO has an important role in development of ED in SHR. Levels of

CO-dependent relaxation of the cavernous tissue were suppressed in adult SHR as compared with WKY rats. In both rat species CO-dependent relaxation of the cavernous tissue was blocked by HO inhibitors. **To our knowledge, the effects of upregulation of HO-1 in the penile tissues, using hemin, have not been previously examined.**

2.2 Hypotheses

1. Metabolism of hemin or HLL after being injected into the rat can be monitored by measuring the changes in circulatory heme levels. A significant positive correlation is present between the circulatory heme levels and the hemin-induced BP changes in SHR and age-matched SD rats.
2. A long-lasting normalization of high BP in adult (12 week-old) SHR can occur after extended continuous hemin administration.
3. Altered expression profiles of HO-1 as well as sGC and PDE-5 in penile tissues can be correlated to low ICP in adult SHR with established hypertension.
4. Hemin-induced upregulation of HO-1 in penile tissues overcomes ED encountered in adult SHR.

2.3 Objectives

1. To monitor the metabolic characteristics of heme or HLL by measuring the changes in circulatory heme levels. Also, to test the presence of correlation between

the circulatory heme levels and the hemin-induced BP changes in SHR and age-matched SD rats.

2. To investigate whether an extended regimen of hemin administration can lead to a long-lasting normalization of high BP in adult (12 week-old) SHR.

3. To investigate whether expression profiles of HO-1 as well as sGC and PDE-5 in penile tissues can be correlated to low ICP in adult SHR with established hypertension.

4. To test the effect of hemin-induced upregulation of HO-1 in penile tissues on ED developed in adult SHR.

3. General Methodology

3.1 Animals

Male SHR and SD rats were purchased from Charles River Laboratories (QC, Quebec). They were housed in an animal care facility at 21ºC with 12-hour light/dark cycles, fed with standard laboratory chow, and had access to drinking water *ad libitum*. The experimental protocol was approved by the University of Saskatchewan Standing Committee on Animal Care and Supply. Hemin or HLL was dissolved in 0.1 M NaOH, titrated to pH 7.4 with 0.1 M HCl, and diluted 1:10 with phosphate buffer solution (PBS) (Ndisang et al., 2003). Care was taken that the volume of NaOH did not exceed 10% of the final hemin solution total volume.

For pump implantation, rats were anesthetized with isoflurane (1.5% in O_2), and then ALZET mini-osmotic pumps (Model 2ML4) were inserted subcutaneously in the subscapular region under sterile conditions. The wound (approximately 1.5 cm) was closed using surgical clips. Prior to implantation, each osmotic pump was filled with hemin solution to deliver 15 mg/kg/day ALZET mini-osmotic pumps (Model 2ML4) were inserted subcutaneously in the subscapular region under sterile conditions. The wound (approximately 1.5 cm) was closed using surgical clips.

It is worth noting that the 2ML4 miniosmotic pump has a total volume of 2 ml and can deliver 90% of this volume in 28 days. Consequently, hemin concentration in the mini-osmotic pump (60-100 mg/ml) far exceeds that injected intraperitoneally (3-5 mg/ml) on a daily basis. This necessitates addition of small volumes of PBS to help in initial dissolving of hemin in NaOH.

3.2 BP measurement

Systolic and mean arterial BPs were determined in conscious rats by means of the standard tail-cuff non-invasive method (Model 29-SSP, Harvard Apparatus, St Laurent, QC, Canada) after acclimatization, before drug administration, and twice weekly during administration period (Ndisang et al., 2003). Post-administration BP measurement was performed in some animal groups. In some animals BP was measured using radiotelemetric BP recording. In this technique, a catheter connected to a radiotelemetry capsule (Data Sciences, Minneapolis, MN) was inserted into the left femoral artery and pushed so that its tip reached the abdominal aorta above the iliac bifurcation for monitoring BP. The capsule containing the transducer and radiotransmitter was positioned in the left flank region subcutaneously. Pressure data were collected with a computer-driven data acquisition system (Data Sciences Inc., St. Paul, MN). The pressure waveform was sampled every 30 s with a 5-s sample duration (Balakrishnan et al., 1998).

3.3 Measurement of serum heme concentration.

Serum heme was measured by the pyridine-hemochromogen method (De Duve 1948). Briefly, at least 0.5 ml of blood obtained from each animal was left to coagulate for 1-1.5 h in special tubes (SST, Vacutainer). Serum was then collected (~0.2-0.25ml) and added to an equivalent amount of pyridine spectrophotometric reagent (99%) in a small tube. The mixture was vortexed for 2 minutes and then 0.1 ml of 0.1M NaOH was added to the solution with vortexing for another 2 minutes. Baseline absorbance was determined using an UV/Visible spectrophotometer

(Ultrospec 3000, Pharmacia Biotech, Cambridge, England). Approximately 10-20 mg of sodium dithionite crystals (reducing agent) were then added to the mixture. Total heme concentration was calculated in micromol/L using the difference in absorbance of the reduced pyridine hemochrome at 557 nm (maximum absorbance) and 541 nm (minimum absorbance) with 20.7 as an extinction coefficient and after applying Beer-Lambert law; [*Difference in absorbance= extinction co-efficient x concentration x path length*] (Maines 1999). Serum heme level was determined prior to drug injection, twice weekly during the injection period, and once per week thereafter. Sera were also collected from all control rats. Standard calibration curve was constituted by using hemin or HLL stock solutions added to sera samples obtained from untreated rats to achieve four different final heme concentrations (1, 3, 10, and 30 micromol/L).

3.4 Western blot analysis

Following animal killing, the mesenteric arterial bed was identified and isolated from the adherent intestine. The isolated vascular tissues were then snap-frozen in liquid nitrogen.

Vascular tissues (mesenteric artery) or penile tissues were homogenized (1:10, w:v) in 10 mM Tris-buffered saline (20 mM Tris-HCl of pH 7.4, 0.25 M sucrose, and 1 mM EDTA) in the presence of a freshly prepared cocktail of protease inhibitors and centrifuged at 10,000g for 10 min at 4^{o}C as previously described (Ndisang et al., 2002). The supernatant was decanted and aliquots of 50 μg of protein were loaded on

a 10% SDS-polyacrylamide gel for sGC, PDE5, or 12.5% for HO-1, HO-2, iNOS, eNOS and VEGF-A. The fractionated proteins were electrophoretically transferred to nitrocellulose paper. Non-specific bindings sites were blocked with 3% non-fat milk dissolved in PBS for 2 h at room temperature. Thereafter, the membranes were incubated overnight with primary antibodies against the following proteins at 4°C: α_1 and β_1 subunits of sGC (US Biologicals, MA, USA) (1:1000 dilution); PDE5 (EMD Biosciences, Inc, Darmstadt, Germany); iNOS (StressGen, Victoria, BC, Canada) (1:1000 dilution); eNOS (StressGen, Victoria, BC, Canada) (1:1000 dilution); HO-1 (Affinity Bioreagents, CO, USA) (1:1000 dilution); HO-2 (StressGen, Victoria, BC, Canada) (1:5000 dilution); VEGF-A (Novus Biologicals, Littleton, CO, USA) (1:100 dilution). After several washes, the nitrocellulose blot was incubated with anti-rabbit IgG or anti-mouse IgG conjugated to horseradish peroxide in 1:5000 dilution (Bio-Rad, CA, USA) for 2 h at room temperature. This was followed by another series of washes with PBS and the immuno-reactivity was visualized by use of enhanced horseradish peroxide/luminol chemiluminescence reagent (Perkin Elmer Life Sciences, Boston, MA, USA). Densitometric scanning and analysis of respective bands of blot were carried out using UN-SCAN-IT software (Silk Scientific, Utah, USA). A monoclonal mouse antibody raised against the structural protein beta-actin (Sigma St Louis, MO, USA) (1:5000 dilution) was used as a control to ascertain equivalent loading. The protein concentration was determined by the Bradford method (Bradford 1976) (Bio-Rad protein assay).

3.5 HO activity assay

The activity of HO was determined as bilirubin production (Llesuy and Tomaro 1994). Liver homogenates from killed rats were prepared using 4 volumes of ice-cold 0.25 M sucrose solution containing phenylmethylsulfonyl fluoride (1mM), EDTA (0.2 mM) and 50 mM potassium phosphate buffer (pH 7.4). The homogenates were centrifuged at 20,000g for 20 min. The supernatant fractions were centrifuged at 150 000Xg for 90 min. The microsomal pellets obtained were washed and resuspended in 20 mM potassium phosphate buffer (pH 7.4), containing 135 mM KCl, 1 mM phenylmethylsulfonyl fluoride and 0.2 mM EDTA to a protein concentration of 10 mg/ml. The 150,000 g supernatant obtained from the microsomal preparation was fractionated by addition of ammonium sulphate (AS), and the 40-60% AS fraction was dissolved in 10 mM potassium phosphate buffer. This preparation was used as biliverdin reductase. Mesenteric arteries harvested from animals treated *in vivo* with hemin were homogenized. They were further incubated for 30 min at 37°C with 50 µl of rat liver biliverdin reductase to convert biliverdin to bilirubin. The amount of bilirubin in each sample was determined spectrophotometrically at 560 nm using Total Bilirubin Kit (Diagnostic Chemicals Limited) and expressed as nmol/mg protein/hour. The protein concentration was determined by the Bradford method (Bradford 1976).

3.6 Measurement of cGMP content

The concentration of cGMP was determined using a radioimmunoassay kit (^{125}I-cGMP-RIA, Amersham International plc, Amersham, UK) as previously

described (Ndisang et al., 2003). Briefly, mesenteric artery tissues were homogenized in 6% trichloroacetic acid at 4°C in the presence of 3'-isobutyl-1-methylxanthine (IBMX) to inhibit phosphodiesterase activity and centrifuged at 2000 g for 15 minutes. The supernatant was recovered and washed three times with water-saturated diethyl ether. The upper ether layer was aspirated and discarded each time after washing while the aqueous layer containing cGMP was recovered, frozen at -20°C and subsequently lyophilized. The dry extract was dissolved in 1 ml of cGMP-assay buffer and the cGMP content was determined using the protocol of the manufacturer and expressed as picomoles of cGMP per mg of protein. The Bradford assay was used to determine the protein concentration (Bradford 1976).

3.7 Morphometric analysis

Morphological examinations were performed as described earlier with some modifications (de Blois et al., 1997). Briefly, rats were anaesthetized and the main branch (superior mesenteric artery) was further identified and cleaned from fat tissue. Following that, the second branch of the mesenteric arterial tree was isolated and cleaned from all adherent tissue. The isolated vascular tissues were then immediately fixed by immersion in 4% paraformaldehyde for 16-18 h. Samples were then incubated in a 30% sucrose solution for 3 days at 4ºC. After embedding in O.C.T. (Optimal Cutting Temperature) compound (Somagen Diagnostics, AB, Canada), sections of 8 micrometer thickness were cut on a cryostat and picked up on poly-l-lysine coated slides. The circumferences of the vessels were measured after obtaining

amplified (×100) images of the sections by a microscope (Olympus 1×70). Diameter (D) was calculated from the equation C= πD, where C is the circumference. Arterial cross-sectional area (CSA) was calculated as CSA= $\pi(r_o^2-r_i^2)$, where r_o is the external radius of the media layer and r_i is the radius of the lumen.

3.8 Determination of drug safety

Parameters selected to assess liver and kidney toxicity were body weight:liver weight ratios (BW/LW), body weight:kidney weight ratios (BW/KW) and plasma enzyme markers of liver and kidney damage. Serum total bilirubin, alanine aminotransferase (ALT), and γ-glutamyltranspeptidase (γGT) were assessed for liver functions, while urea and creatinine plasma levels were assessed for kidney functions. Body weights (BW), in grams, were recorded prior to sacrifice by decapitation. After sacrifice, the liver and kidneys were rapidly removed and placed in ice cold PBS, trimmed of fat and connective tissue and then liver and kidney weights in grams) were recorded. Immediately on sacrifice, whole blood was collected in chilled 50 ml beakers containing 70 µl of an anticoagulant solution that was made up with 10.5 mg tri-potassium ethylenediaminetetraacetic acid and 0.014 mg potassium sorbate. The whole blood was then centrifuged at 2000 g at 4°C for 10 minutes. The resulting plasma was immediately removed by Pasteur pipette, dispensed into sample tubes and assayed for bilirubin, ALT, γGT, urea and creatinine using established colourimetric assays.

3.9 Intracavernous pressure response assessment

At the end of the 3-week injection period ICP responses were elicited by electrical stimulation of the cavernous nerve in some anesthetized rats, as previously described (Sato et al., 2001). Briefly, rats were anesthetized with xylazine (10 mg/kg, i.p.) and ketamine (90 mg/kg, i.p.), and maintained at 37°C. The tail artery was cuffed to record BP, and a 21-gauge needle was inserted into one of the corpus cavernosum of the penis to record intracavernous pressure (ICP) via a pressure transducer (Elcomatic 750, Elcomatic, Glasgow, UK) and recorded on a pressure polygraph (Pressure Polygraph 7, Grass Instruments, MA, USA). The cavernous nerve (CN) was exposed at the lateral side of the prostate and mounted on a bipolar platinum electrode connected to an electrical stimulator (AMS 2100, Phymep, France). For each animal, electrical stimulations of the CN (square-wave pulses of 1 ms, duration of 1 min, 6 V) at different frequencies (1, 2, 3, 4, 5, and 10 Hz) were performed in a randomized manner and repeated twice to establish frequency-response curves. The ICP responses elicited by electrical stimulations were quantified by calculating the ratio ΔICP (mmHg)/MAP (mmHg) x 100, with ΔICP being the difference between ICP in the flaccid state, i.e., before stimulation, and ICP during the plateau phase and with MAP being the mean arterial pressure during the plateau phase. This ratio accounts for the influence of the systemic BP on the amplitude of ICP increase during the plateau phase (Sato et al 2001). At the end of the experiments, rats were killed by an anesthetic overdose.

3.10 Statistical Analysis

All data were expressed as means ± SEM from at least three independent experiments performed in duplicates unless otherwise stated. Statistical analyses were done using unpaired Student's *t*-test, analyses of variance in conjunction with Newman-Keuls test and analyses of variance for repeated measures where appropriate. Group differences at the level of $p<0.05$ were considered statistically significant.

4. Results

4.1 Monitoring circulatory heme levels after hemin therapy for SHR

A total of 64 animals aged 12, 24, and 33 weeks were used, of which 42 were SHR and 22 were SD rats. SHR 12 weeks old (n=20) weighed 278 ± 4.5 g and age-matched SD rats (n=10) 289 ± 6.1 g. SHR 24 weeks old (n=6) and age-matched SD rats (n=6) weighed 309 ± 5.4 and 342 ± 4.7 g, respectively. SHR 33 weeks old (n=6) and age-matched SD rats (n=6) weighed 345 ± 6.1 and 414 ± 4.3 g, respectively. Hemin or HLL (15 mg/kg) was injected (i.p.) daily into 12 weeks old SHR (n=10 for each group) for 13 days. The same treatment regimen was applied to 6 SD rats and 6 SHR older than 20 weeks for 5 days. The remaining 10 SHR and 10 SD rats of 12 weeks old, 3 SHR and 3 SD rats 24 weeks old, and 3 SHR and 3 SD rats 33 weeks old were used as untreated control. BP was measured in all animals using tail-cuff method.

4.1.1 Development of a standard calibration curve for measurement of serum heme concentration *in vitro*

A linear relationship was obtained between the absorbance difference of reduced pyridine hemochrome and hemin at known concentrations. There was no significant difference in the quantitation of heme levels between hemin and HLL solutions at the same concentrations (1, 3, 10, and 30 micromol/L) *in vitro* (Figure 4.1).

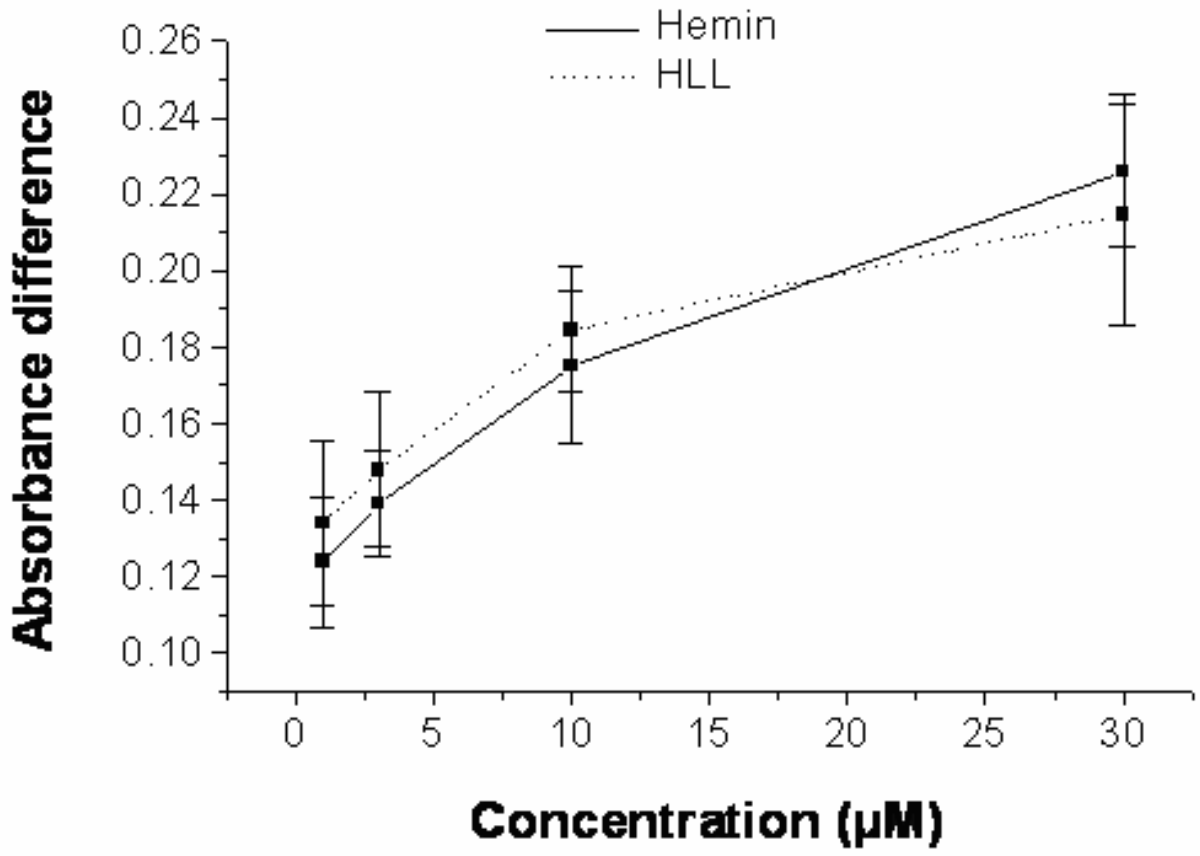

Figure 4.1 Standard calibration curve of absorbance difference (557 and 541 nm) of the reduced pyridine hemochromogen after addition of different concentrations of hemin and HLL solutions to a mixture of pyridine spectrophotometric reagent 99% and sodium dithionite. A near linear relationship is observed between absorbance difference and concentration (micromol/L) with no significant difference between hemin and HLL. Data is presented as mean of 60 trials.

4.1.2 Effects of 13-day hemin/HLL treatment regimen on young SHR (12 weeks old)

Systolic BP level in SHR was significantly higher ($p<0.05$) than in age-matched SD rats at all age groups (Figure 4.2). Baseline serum heme levels were either undetectable or < 1 micromol/L in all rats. After 13 days of daily injections of hemin or HLL, systolic BP of 12 week old SHR significantly decreased by 15.5 ± 2.3% or 16.4 ± 1.8%, respectively ($p<0.05$). However, no difference was observed in the anti-hypertensive potency between hemin and HLL (Figure 4.3A). Immediately after the beginning of daily injections of either hemin or HLL, serum heme level increased significantly from nearly undetectable to 4.3 ± 0.3 or 4.7 ± 0.5 micromol/L, respectively. This elevated serum level of heme remained throughout the injection period (Figure 4.3B). There was no significant difference in the elevated serum heme levels induced by hemin and HLL ($p>0.05$).

4.1.3 Effects of 5-day hemin/HLL treatment regimen on SHR and SD rats older than 20 weeks

Short period (5 days) treatments of SHR and SD rats older than 20 weeks did not change BPs although the basal levels of BP in these two strains of rats were significantly different ($p<0.01$, n=6 for each group) (Figure 4.4A). Immediately after the beginning of daily injections of either hemin or HLL, serum heme level increased significantly from nearly undetectable to 4.7 ± 0.43 or 4.2 ± 0.7 micromol/L in SHR and 4.6 ± 0.2 or 4.8 ± 0.7 micromol/L in the age-matched SD rats, respectively (Figure 4.4B). When administration of either hemin or HLL was discontinued, serum

heme level decreased gradually in all rats over a period of 12 days to reach the baseline levels.

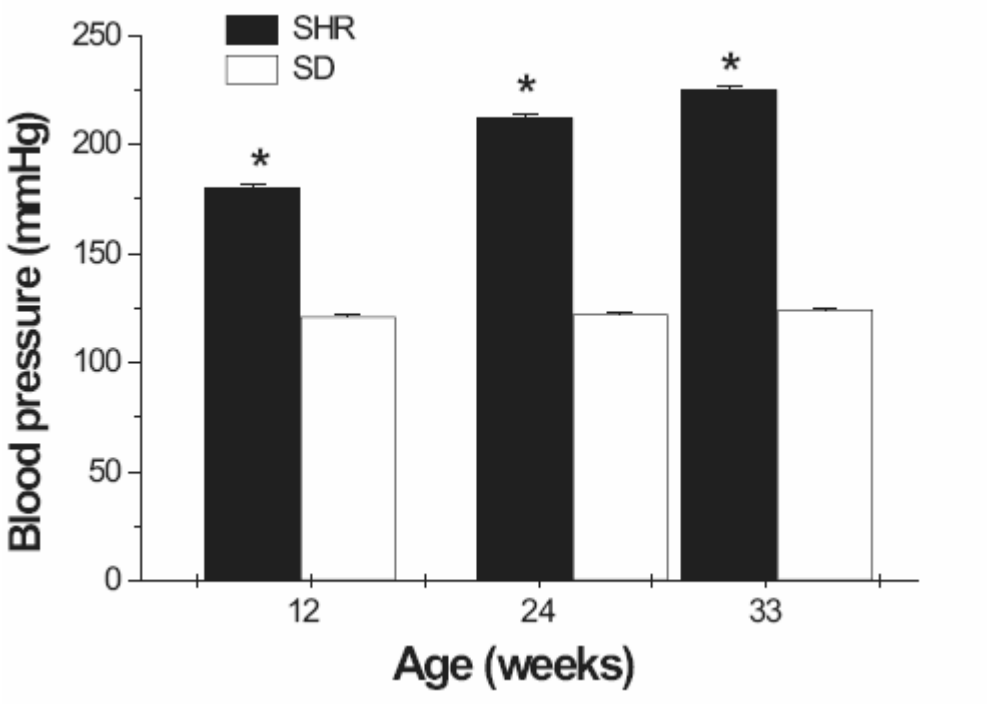

Fig. 4.2 BP of SHR and SD rats. Systolic BP of SHR and SD rats of different ages showing significant difference (*$p<0.05$). n = 10 for each group of 12 week old SHR and SD rats. n = 3 for each group of 24 week and 33 week old SHR and SD rats.

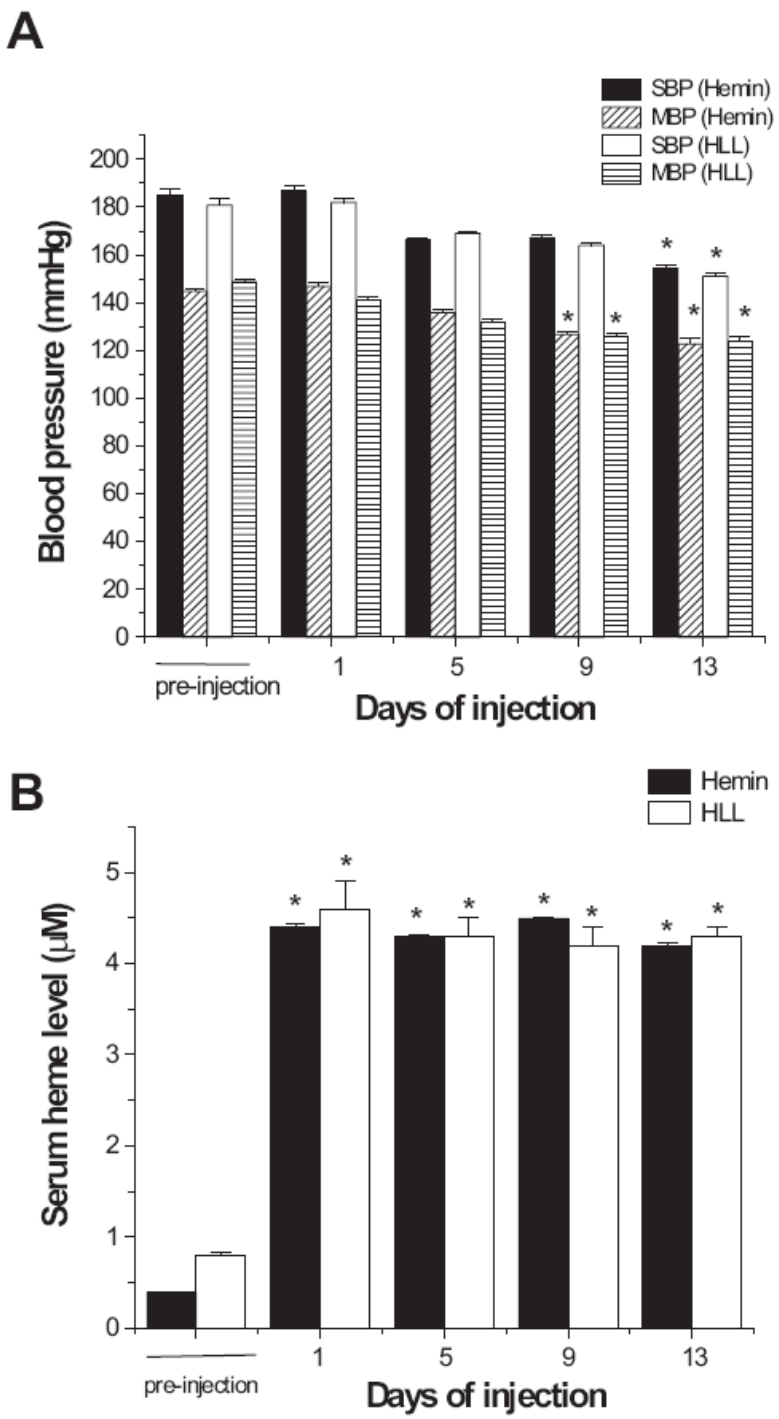

Figure 4.3 Comparison of the effects of 13-day hemin HLL treatments on BPs and serum heme levels of 12 week old SHR (n=10, each group). A) BP lowering effects of hemin and HLL treatments (*$p<0.05$). No significant difference in BP lowering effect was observed between hemin and HLL treatments. SBP, systolic BP; MBP, mean BP. B) Elevation of serum heme levels after injection of hemin or HLL (*$p<0.05$ vs. basal level). No significant difference in serum heme levels was observed between hemin and HLL treatments.

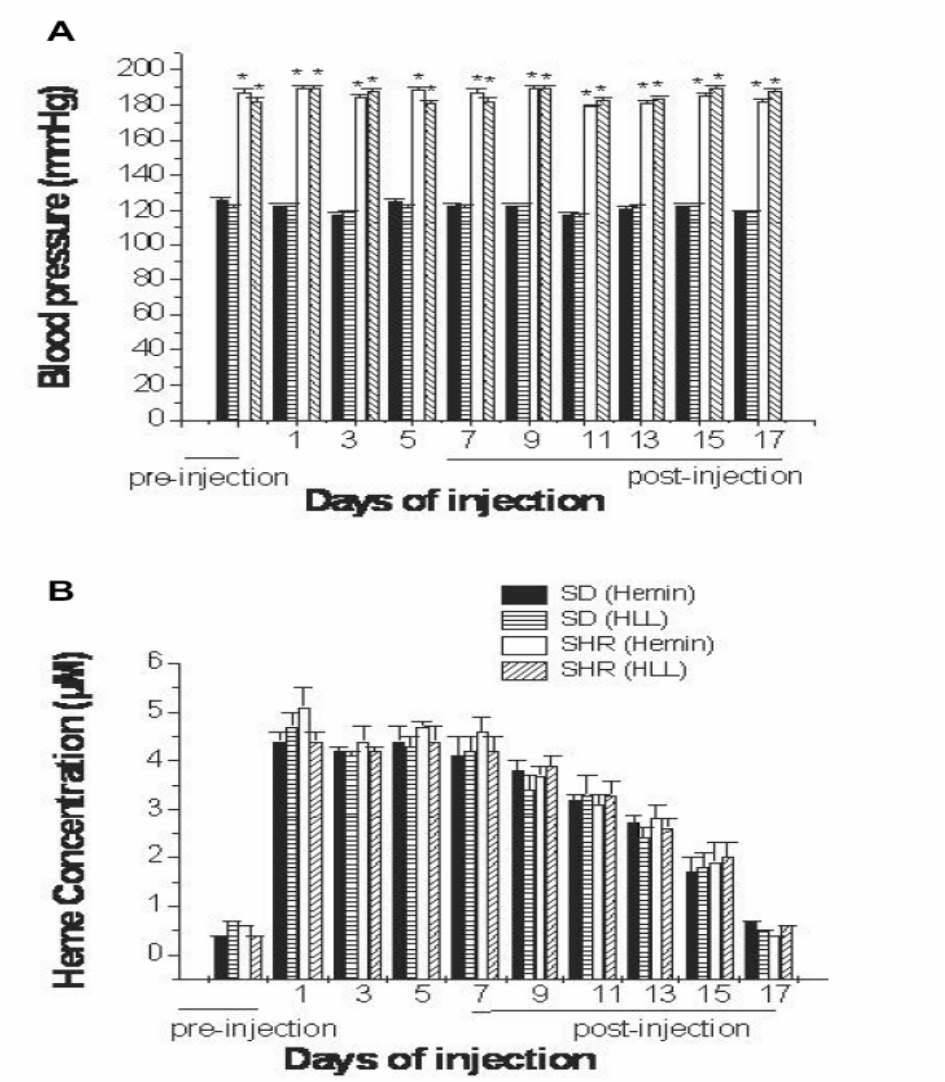

Fig. 4.4 Comparison of the effects of 5-day hemin and HLL treatments on BPs and serum heme levels of SHR (n = 6) and SD rats (n = 6) older than 20 weeks. A) Acute 5-day injections of hemin or HLL did not alter systolic BPs of SHR and age-matched SD during hemin or HLL injection period. B) Serum heme levels in SHR and SD rats treated with hemin or HLL were elevated significantly during the 5-day daily injection period ($p<0.05$ vs basal level) and gradually dropped to baseline values after treatment stoppage.

4.2 Effect of hemin protocol on BP of adult SHR and its underlying mechanisms

A total of 150 12-week old male SHR (n=80) and Sprague Dawley (SD) rats (n=70) were used in this study. Hemin was administered using Alzet osmotic minipumps (Alzet, California, USA) for 21 consecutive days (the hemin protocol) to 12 week old SHR (n=20) and SD rats (n=20). Prior to implantation, each osmotic pump was filled with hemin solution to deliver 15 mg/kg/day (Ndisang et al., 2003). ALZET osmotic pump model delivers at a rate of 2.5 µl/h for the tested period. At the end of the 21-day pump infusion period, all pumps were explanted. In another experiment hydralazine (45 mg/kg/d) (Hale et al., 2001) was given orally to 12 weeks old SHR (n=20) and age-matched SD rats (n=10) for 21 days.

Sham control groups, composed of 12 week old SHR and SD rats were infused with vehicle solution of 0.9% NaCl (2 ml) using osmotic minipumps (n=20 per group) for 21 consecutive days. The rest of the animals (20 SHR and 20 SD rats) were left untreated as age controls. BP was measured in all animals using the tail-cuff method. In some animals BP was assessed using radiotelemetry. Serum heme was determined prior to hemin protocol, during infusion period and following treatment termination. Protein expression levels using Western blot analysis, HO activity and cGMP content were determined in mesenteric tissues. Morphological assessment was performed using main branch of mesenteric arteries. Assessment of hemin safety was performed as described in Section 3.8.

4.2.1 Normalization of BP in adult SHR during and after the hemin protocol

Systolic BP of adult SHR was lowered to the normotensive level 2 weeks after the start of the hemin protocol (203 ± 2.5 vs. 123 ± 1.9 mmHg, n=20). However, the hemin protocol had no effect on age-matched normotensive SD rats (121 ± 1.8 vs.118 ± 1.2 mmHg, n=20) (Fig. 4.5A). On the other hand, systolic BP of vehicle-treated SHR remained in hypertensive range at the end of 3-week administration (211± 2.8 vs. 204 ± 2.2 mmHg, n=20). Fig. 4.5B shows the day-to-day change in systolic BP of hemin-treated SHR throughout the hemin protocol duration.

The anti-hypertensive effect of the hemin protocol was sustained after the removal of hemin pumps for 9 months (140 ± 2.6 mmHg) (Fig. 4.5C). By the 10^{th} month post-hemin protocol, systolic BP of SHR reached 148 ± 2.3 mmHg while that of untreated SHR was 228 ± 2.3 mmHg ($p<0.001$) (Fig. 4.5C).

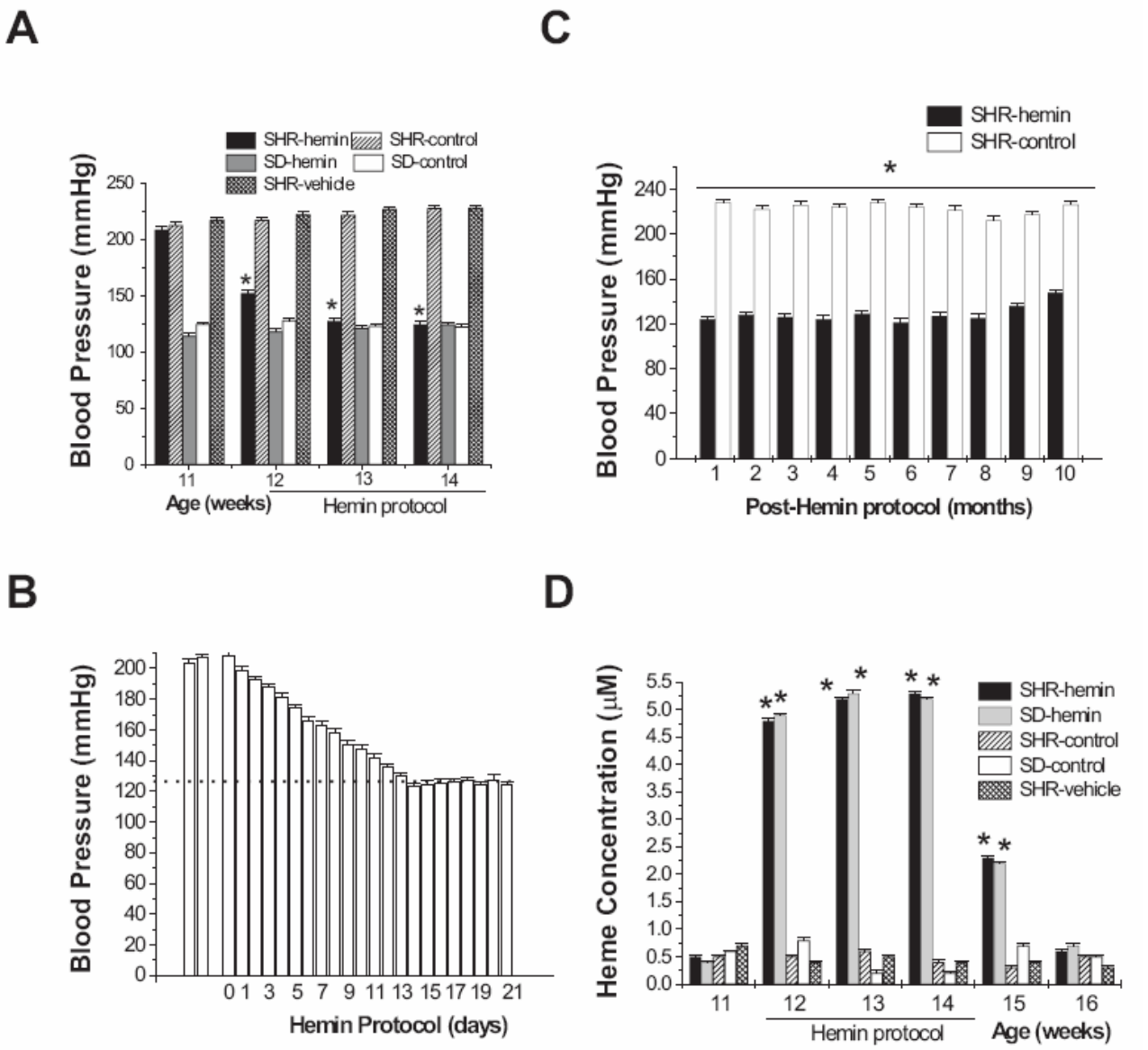

Fig 4.5 Systolic BP and serum heme levels during and after the hemin protocol. A) Changes in systolic BP in different animal groups during the 3-week hemin protocol. (n=20 for each group; *p<0.001 vs. pre-treatment or SHR control/SHR-vehicle). B) Changes in systolic BP in adult SHR during the 3-week hemin protocol (n=20). C) Normalization of BP in hemin-treated SHR after the hemin pump removal. n=10 for each group. * p<0.001 vs. age-matched control SHR. D) Serum heme levels before, during, and after hemin treatment in different groups of animals. n=20 for each group. p<0.05 vs. untreated rats. SHRV, SHR treated with 0.9% NaCl as the vehicle control.

4.2.2 Changes in serum heme levels, expression of HO-1 proteins and HO-activity during and after the hemin protocol

Immediately after the start of the hemin protocol, serum heme levels of SHR (n=20) and SD rats (n=20) increased significantly from a nearly undetectable baseline to 4.8 ± 0.1 μM and 5.1 ± 0.1 μM, respectively (Fig. 4.5D). Two weeks after the removal of hemin pumps serum heme levels returned gradually to the baseline. Serum heme levels of saline-treated or untreated SHR and SD rats (n=10 per group) did not change significantly during and after the 3-week treatment/observation period.

The basal expression level of HO-1 protein in mesenteric arteries was significantly higher in adult SHR than that in age-matched SD rats. At the end of the 3-week hemin protocol, HO-1 expression was significantly upregulated in both SHR and SD rats, with the former being significantly greater ($p<0.01$) (Fig. 4.6A). In contrast, no difference was detected in the expression levels of the constitutive HO-2 proteins in the mesenteric artery of SHR and SD rats with or without hemin treatment (Fig. 4.6B). Consistent with HO-1 expression change, the 3-week hemin protocol significantly increased total HO activity in the mesenteric arteries of adult SHR (Fig. 4.6C). Although an increase in HO activity was also detected in hemin-treated SD rats, the amount of increment in the hemin-treated SHR was significantly greater (54% in SD rats vs. 300% in SHR, $p< 0.05$).

For the purpose of comparison, adult SHR were also treated for 3 weeks with hydralazine. Hydralazine also significantly normalized systolic BP of adult SHR 3 weeks after continuous oral therapy (184 ± 2.3 vs. 126 ± 2.3 mmHg, n=20) (Fig. 4.7A). The anti-hypertensive effect of hydralazine treatment, however, only lasted

about 2 weeks after the termination of hydralazine administration (134 ± 3.1 mmHg) (Fig. 4.7A). Thereafter, systolic BP of these SHR gradually climbed up. At week 4 after hydralazine treatment, systolic BP of hydralazine-treated SHR was restored to the pre-treatment level (184 ± 2.3 vs. 178 ± 3.2, n=10).

The acute anti-hypertensive effect of hydralazine was not related to the function of the HO/CO system since at the end of the 3-week hydralazine treatment the expression of HO-1 did not change significantly in mesenteric arteries compared to with that of non-treated age-matched SHR (Fig. 4.7B). Hydralazine treatment also did not alter the expression levels of HO-2 in mesenteric arteries of adult SHR (Fig. 4.7C).

Systolic BP measurements from telemetric BP monitoring of animals (n=3 per group) followed a similar pattern as those obtained using the tail-cuff method (Fig 4.8).

Hemin-induced upregulation of HO-1 in SHR remained for 9 months after the removal of the hemin pumps ($p<0.05$ vs. control SHR) (Fig. 4.9A). Similarly, total HO activity remained significantly higher in 55 week-old hemin-treated SHR than age-matched untreated SHR (Fig. 4.9B).

4.2.3 Changes in sGC expression, cGMP content and PDE-5 expression during and after the hemin protocol

Adult SHR showed comparable levels of cGMP content and sGC proteins in vascular tissues with those of age-matched SD rats (Fig. 4.10A, B). Expression level of sGC protein and content of cGMP in vascular tissues of SHR, but not those of

hemin-treated SD rats, were significantly increased at the end of 3-week hemin protocol (Fig. 4.10A, B). Adult SHR had a significantly higher PDE5 protein expression level in vascular tissues than did SD rats. The hemin protocol significantly downregulated the expression of PDE5 in vascular tissue of SHR, but not that of hemin-treated SD rats, at the end of 3 week treatment period (Fig. 4.10C).

Nine months after the removal of the hemin pumps, sGC protein expression level and the content of cGMP in mesenteric arteries of adult SHR remained significantly higher than that of age-matched control SHR or hemin-treated SD rats ($p<0.05$) (Fig. 4.11A, B). Down-regulated expression of PDE-5 by the hemin protocol in the mesenteric artery of SHR also remained for 9 months after hemin pump removal (Fig. 4.11C).

4.2.4 Changes in expression level of iNOS and eNOS proteins

Basal expression level of iNOS proteins in the mesenteric artery of adult SHR was significantly higher than that of age-matched SD rats (Fig. 4.12A). In contrast, no difference was detected in basal expression of eNOS proteins between the mesenteric artery of adult SHR and SD rats (Fig. 4.12B). At the end of the 3-week hemin protocol, there was no difference in the expression levels of iNOS or eNOS proteins between treated or non-treated age-matched SHR (Fig. 4.12).

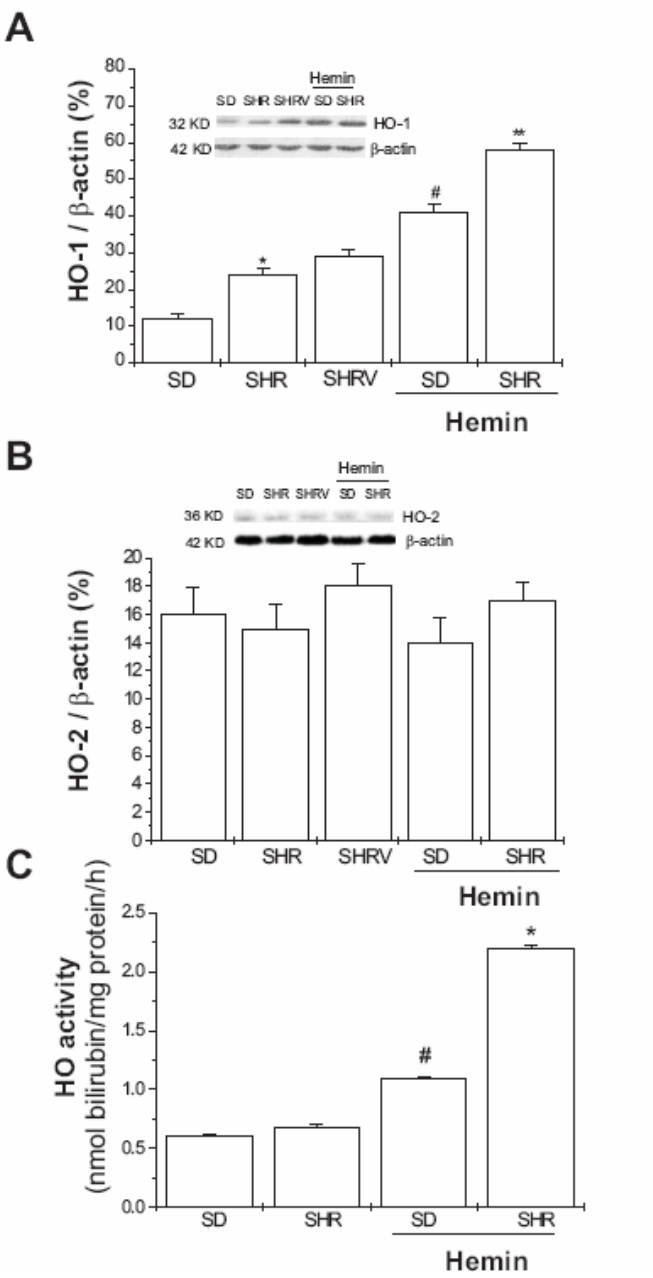

Figure 4.6. Expressions of HO-1 and HO-2 proteins and total HO activity in the mesenteric arteries in different age-matched animal groups at the end of 3-week hemin protocol. A) Representative Western blot (inset) and summary (bottom) of relative abundance expression levels of HO-1 proteins. *p<0.05 vs. SD rats; **p<0.01 vs. all other groups. # p<0.05 vs untreated SD rats, n=10 for each group. B) Representative Western blot (inset) and summary (bottom) of relative abundance expression levels of HO-2 proteins.. **C.** Total HO activity in the mesenteric arteries from different age-matched animal groups. *p<0.05 vs. all other groups, # p<0.05 vs untreated SD rats, n=10 for each group.

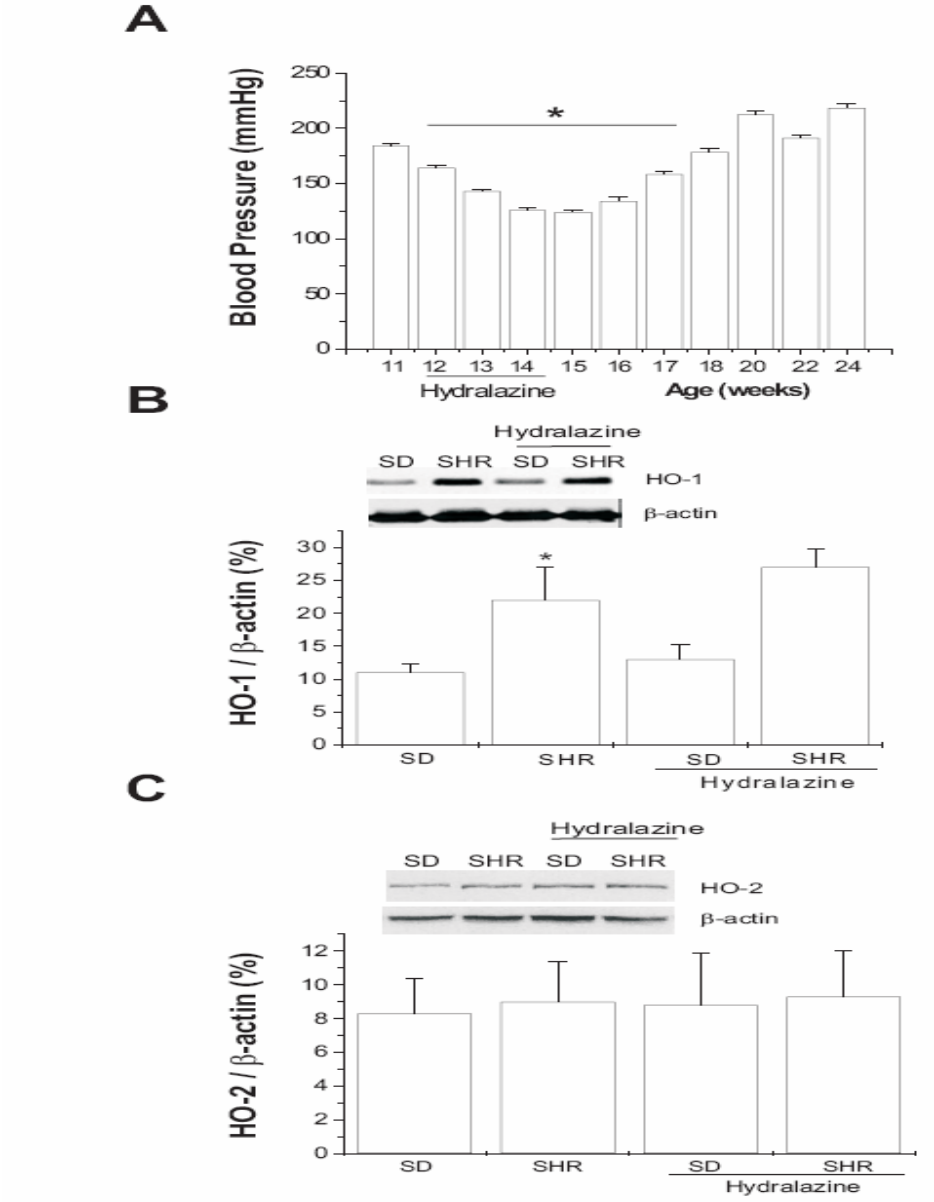

Figure 4.7. Anti-hypertensive effects of hydralazine on adult SHR. A) Normalization of BP and its rebounded increase in hydralazine-treated SHR during and after hydralazine treatment, respectively. n=10, $p<0.05$ vs. pre-treatment levels. B) Representative Western blot (inset) and summary (bottom) of relative abundance expression levels of HO-1 proteins in the mesenteric arteries of SHR and SD at the end of 3-week hydralazine treatment or that of age-matched untreated animals. *$p<0.05$ vs. SD rats; n=10 for each group. C. Representative Western blot (inset) and summary of relative abundance expression levels of HO-2 proteins in the mesenteric arteries of SHR and SD at the end of 3-week hydralazine treatment or that of age-matched untreated animals. n=10 for each group.

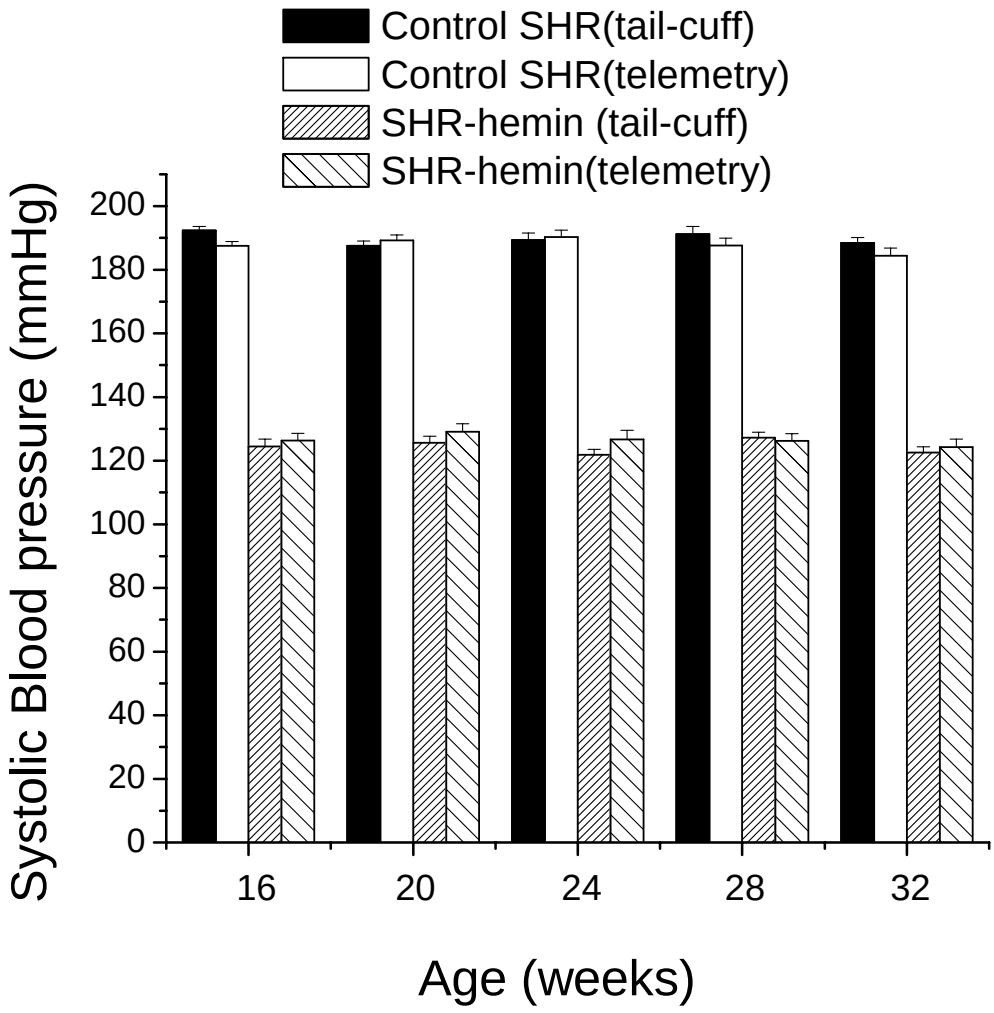

Figure 4.8 Comparison between systolic BP measurements recorded from all animal groups, following hemin protocol stoppage (animals older than 15 weeks), using tail-cuff versus radiotelemetry, n=3 per group. There was no significant difference in systolic BP measurements between tail-cuff and radiotelemetry in the same group.

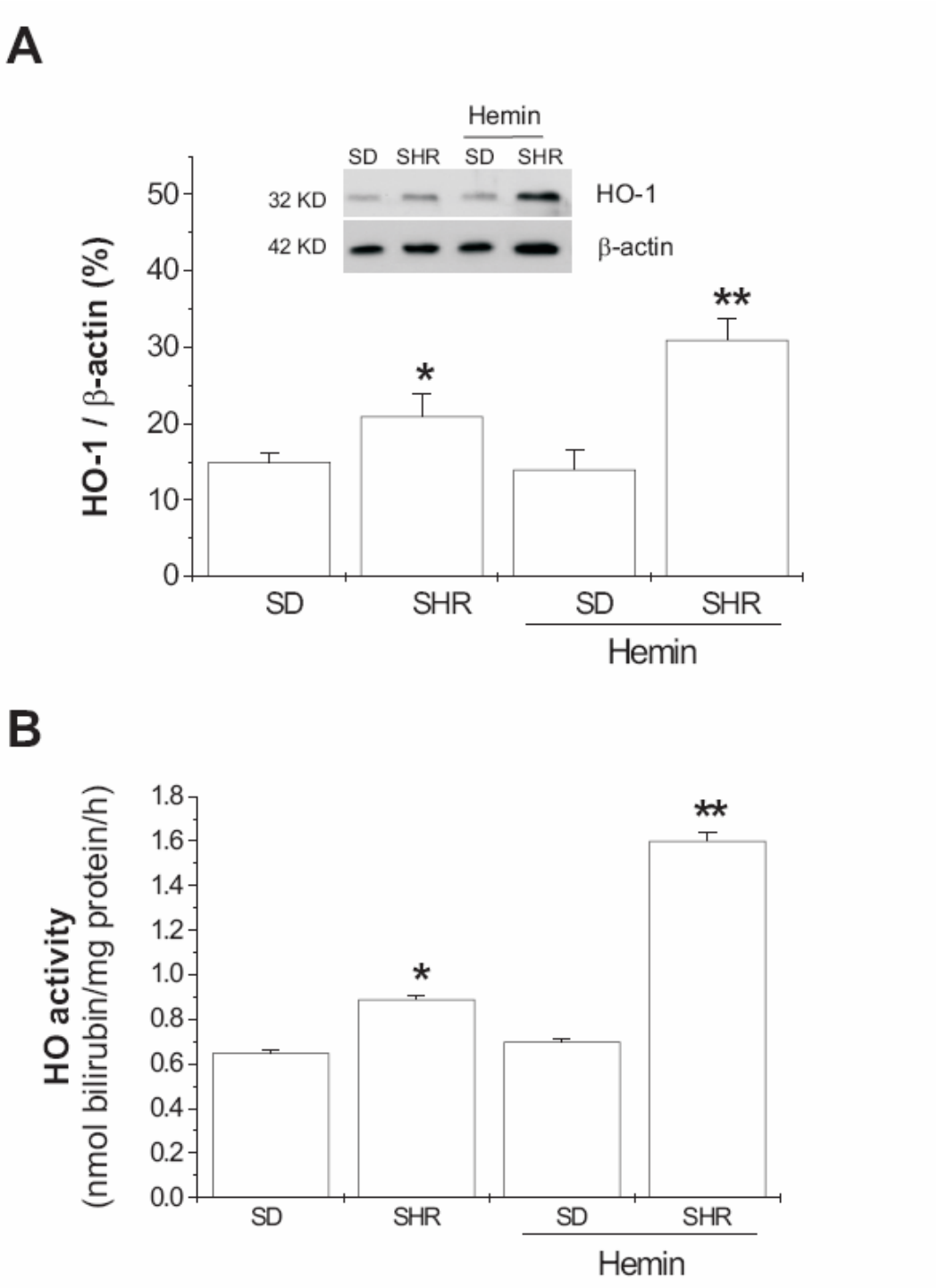

Figure 4.9 Expressions of HO-1 protein and total HO activity in the mesenteric arteries of old SHR and SD rats 9 months after the removal of hemin pumps. A) Representative Western blot (inset) and the summary of relative abundance expression levels of HO-1 proteins. *p<0.05 vs. untreated SD rats; **p<0.05 vs all other groups; n=10. B) HO activity changes in the mesenteric arteries of hemin-treated adult SHR. *p<0.05 vs. untreated SD rats; **p<0.05 vs all other groups; n=10 for each group.

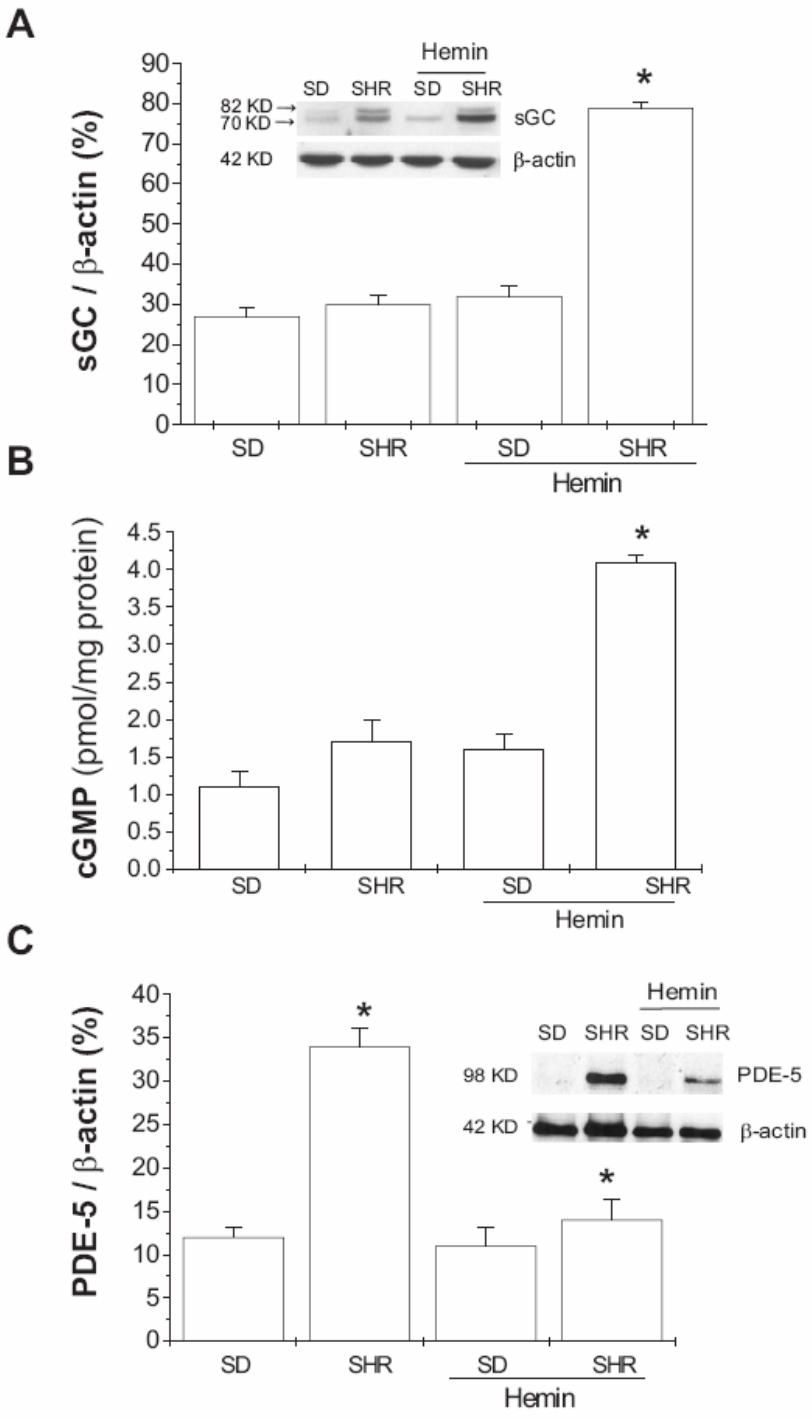

Figure 4.10 Expression of sGC and PDE-5 proteins and cGMP content in the mesenteric arteries of 15 week-old SHR and SD rats at the end of 3-week hemin protocol. A) Representative Western blot (inset) and summary of relative abundant levels of sGC proteins. *$p<0.05$ vs. all other groups; n=10 for each group. B) The hemin protocol increased cGMP content in adult SHR. *$p<0.05$ vs. all other groups; n=10 for each group. C) Representative Western blot (inset) and summary of relative abundant levels of PDE-5 proteins. *$p<0.05$ vs. all other groups; n=10 for each group.

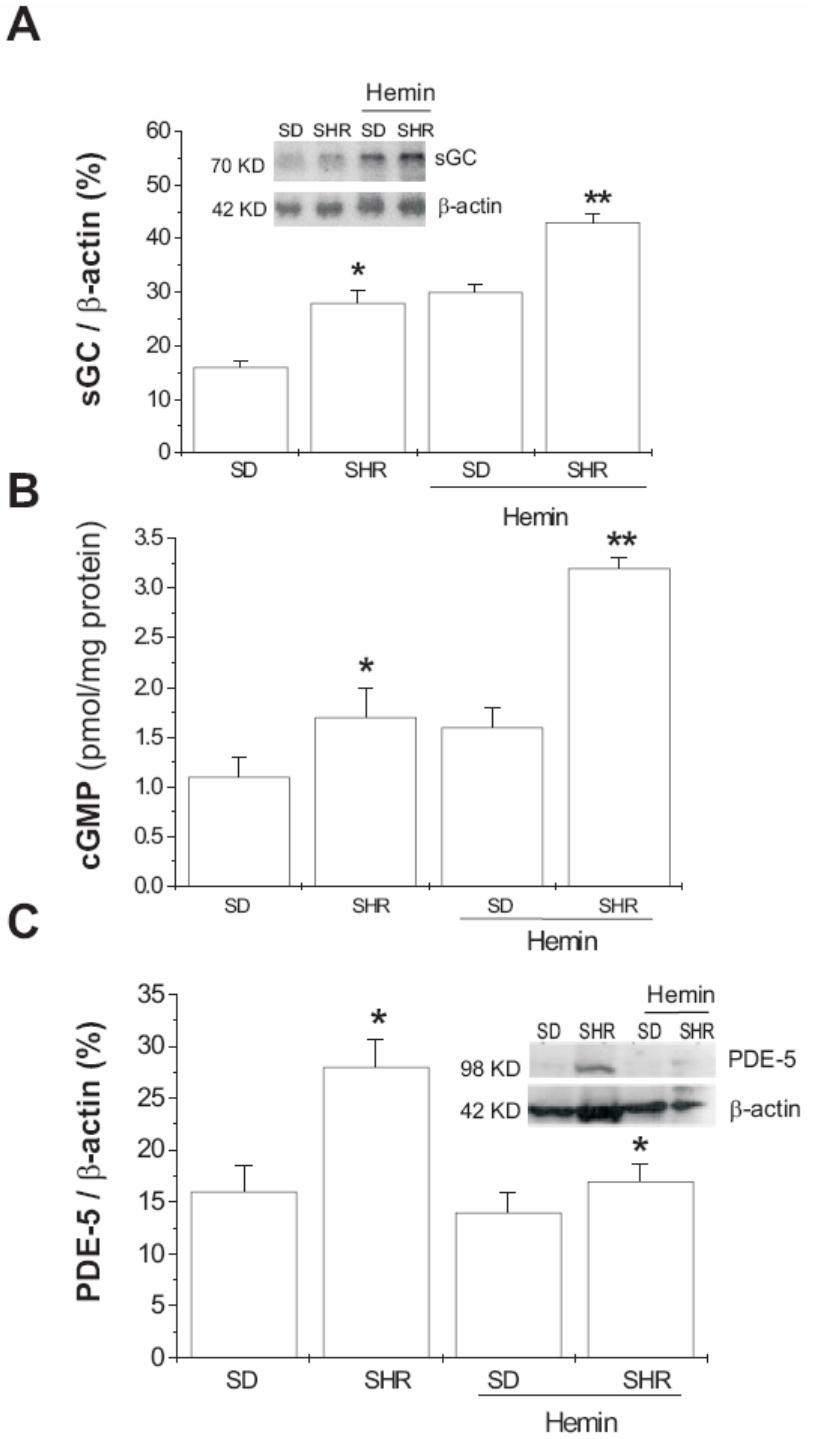

Figure 4.11 Expression of sGC and PDE-5 proteins and cGMP content in the mesenteric arteries of adult SHR and SD rats 9 months after the removal of hemin pumps. **A.** Representative Western blot (inset) and the summary of relative abundant levels of sGC proteins. *p<0.05 vs. control SD, **p<0.05 vs all other groups; n=10 for each group. **B.** The hemin protocol increased the cGMP content in adult SHR. *p<0.05 vs. control SD, **p<0.05 vs all other groups; n=10 for each group. **C.** Representative Western blot (inset) and summary of relative abundant levels of PDE5 proteins. *p<0.05 vs. all other groups;n=10 for each group.

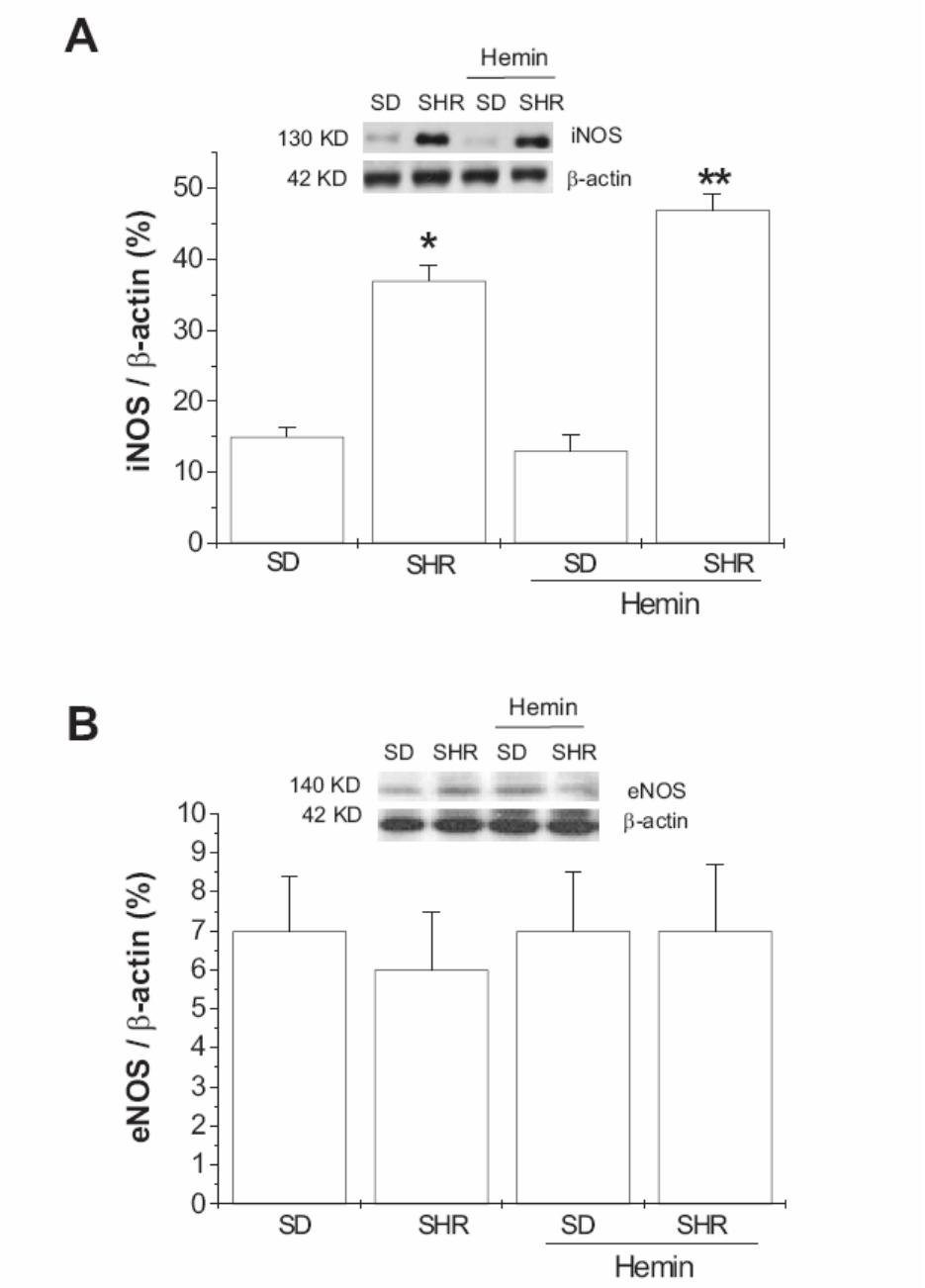

Figure 4.12 Expressions of iNOS and eNOS proteins in the mesenteric arteries of adult SHR and SD rats. A) Representative Western blot (inset) and summary of relative abundant levels of iNOS proteins. *p<0.05 vs. untreated SD rats; **p<0.05 vs hemin-treated SD rats;n=10 for each group. B) Representative Western blot (inset) and summary of relative abundant levels of eNOS proteins. n=10 for each group. Hemin protocol didn't significantly affect expression of either iNOS or eNOS in the mesenteric arteries of SHR rats.

4.2.5 Vascular remodeling in SHR

Significant eutrophic inward remodeling of small mesenteric arteries (main branch) of adult SHR was confirmed with decreased lumen diameter and increased wall/lumen ratio in comparison with age-matched SD rats (Table 4.1; Fig 4.13). The hemin protocol resulted in significant reversal of eutrophic inward remodeling of the mesenteric arteries of SHR at the end of the 3-week treatment. Arterial lumen sizes, wall media, wall/lumen ratio and CSA of hemin-treated SHR were reversed to the levels of those of age-matched normotensive SD rats (Table 4.1; Fig 4.13A). The reversed eutrophic inward remodeling of mesenteric arteries of SHR was sustained 9 months after the removal of hemin pumps whereas the age-matched 55-week old untreated SHR still exhibited significant eutrophic inward remodeling in comparison to age-matched SD rats (Table 4.1; Fig 4.13B).

Table 4.1. Morphological changes of the mesenteric arteries (main branch) of hemin-treated and untreated SHR

Group (age)	n	Lumen diameter (mm)	CSA (mm^2)	Media (μm)	Media/Lumen (%)
Control SHR (15-weeks)	10	0.53±0.02	0.17±0.06	84.7±2.4	15.9±0.2
Hemin-treated SHR(15 week-old)	10	0.68±0.03*	0.18±0.02	82.7±1.8	12.1±0.5*
SD (15 week-old)	10	0.74±0.04**	0.18±0.05	79.3±2.2	10.7±0.4**
Control SHR (55 week-old)	10	0.52±0.05	0.17±0.04	85.7±1.7	16.4±0.1
Hemin-treated SHR(55 week-old)	10	0.64±0.03*	0.18±0.05	83.4±3.2	13±0.1*
SD rats (55 week-old)	10	0.71±0.03**	0.18±0.03	78.4±2.4	11±0.2**

Note: Chronic hemin treatment significantly decreased eutrophic remodelling of the mesenteric artery in adult SHR. The lumen diameter of hemin-treated animals was similar to those of age-matched normotensive SD and significantly different from untreated SHR. *$p<0.05$ vs. SHR; **$p<0.05$ vs. untreated age-matched control SHR.

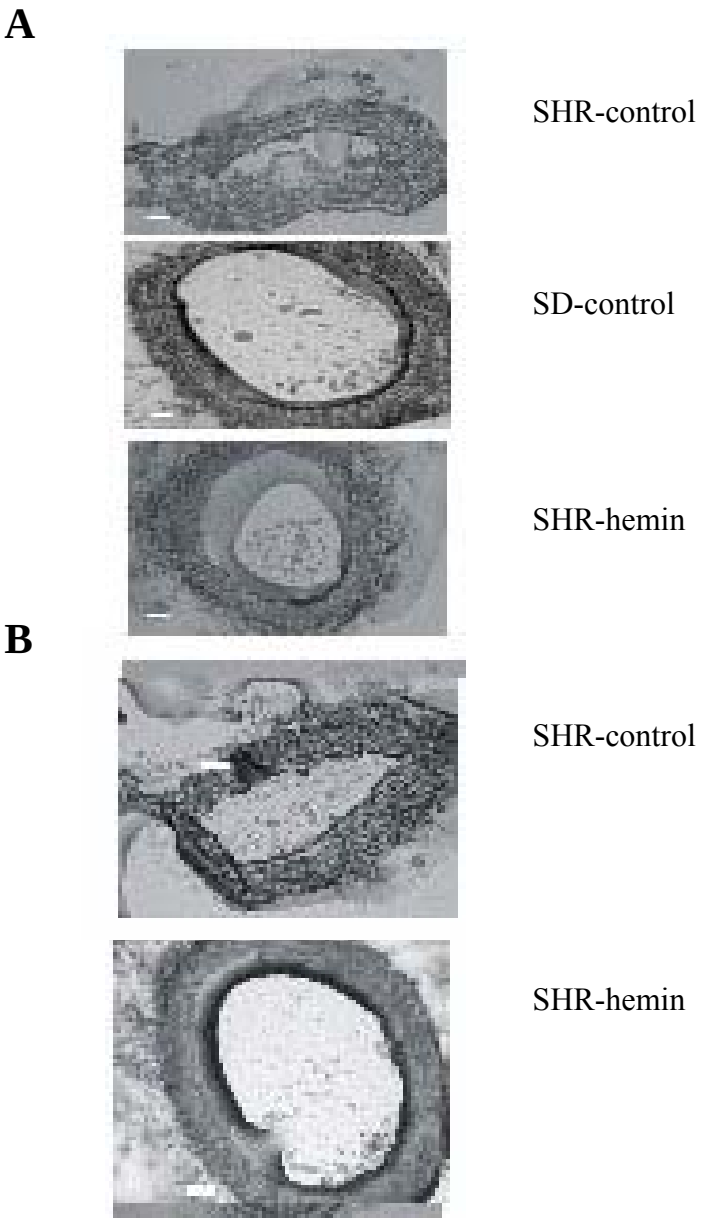

Fig 4.13 Morphological changes in mesenteric arteries of hemin-treated and untreated rats. A) Arterial lumen sizes of the mesenteric artery were greatly reduced in 15-week old SHR. Hemin treatment restored arterial lumen size of SHR to similar dimensions as age-matched SD rats. B) At 55 weeks of age, morphometric parameters in adult SHR were still notably different from untreated SHR. [Magnification 10X; scale bar 50 μm]

4.2.6 Changes in the expression of VEGF-A

Basal expression level of VEGF-A proteins in vascular tissues of SHR was higher than that of age-matched SD rats. Reduced expression of VEGF-A in the mesenteric artery of SHR after a 3-week hemin protocol was observed (Fig. 4.14A). Nine months after the removal of hemin pumps, expression level of VEGF-A proteins in mesenteric arteries of SHR was still significantly lower than that of age-matched untreated SHR rats ($p<0.05$, Fig. 4.143B). Hemin treatment also significantly lowered the expression of VEGF-A proteins in mesenteric arteries of SD rats at the end of the 3-week hemin protocol as well as 9 months later in comparison with that of age-matched untreated SD rats (Fig. 4.14).

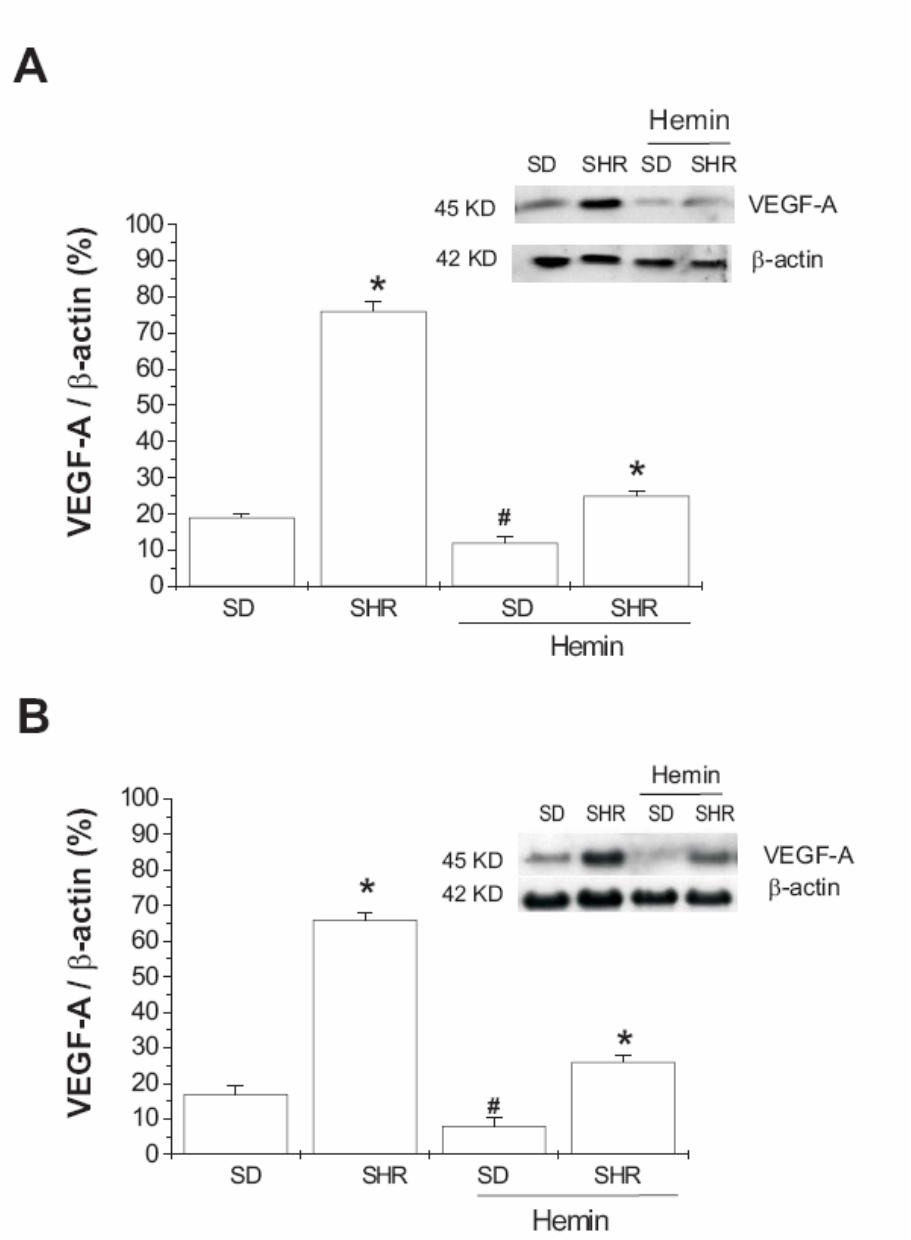

Figure 4.14. Expression of VEGF-A proteins in the mesenteric arteries of SHR and SD rats induced by the hemin protocol. A) Representative Western blot (inset) and summary of relative abundant levels of VEGF-A proteins in 15 week old SHR and SD rats at the end of 3-week hemin protocol. *p<0.01 vs. all other groups;#p<0.01 vs. untreated SD rats. n=10 for each group. B) Representative Western blot (top) and the summary (bottom) of relative abundant levels of VEGF-A proteins in 55 week-old SHR and SD rats 9 months after the removal of hemin pumps. *p<0.01 vs. all other groups; **p<0.01 vs. untreated SHR, n=10 for each group. Hemin protocol significantly affected expression of VEGF in the mesenteric arteries of SD rats, $^{\#}$p<0.05 vs untreated SD rats, n=10 per group.

4.2.7 Safety of the hemin protocol

Growth retardation of SHR was significant since body weights of SHR older than 10 weeks were significantly lighter than that of age-matched SD rats (Table 2). The 3-week hemin protocol did not change body weights of the treated SHR in comparison to that of age-matched untreated SHR ($p>0.05$) (Table 2).

Plasma ALT and γGT are important markers of hepatotoxicity. Comparable plasma levels of ALT were found in 15 week old untreated SHR (n=7) and age-matched treated SHR at the end of the 3-week hemin protocol (n=7) (44.1 ± 2.1 vs. 47.9 ± 3.5 IU/L). Plasma γGT level in untreated 15 week old SHR (n=7) was 43.4 ± 5.2 IU/L, while it was 49.3 ± 3.8 IU/L in age-matched treated SHR at the end of the 3-week hemin protocol (n=7) ($p>0.05$). Serum total bilirubin levels were within the normal range (0.2-1.2 mg/dl) in treated SHR at the end of 3-week hemin protocol (n=7) (0.9 ± 0.02 mg/dl), but significantly greater than that in age-matched 15 weeks old untreated SHR (n=7) (0.4± 0.03 mg/dl, $p<0.05$). Furthermore, in untreated 15 week old SHR rats (n=10) body weight/liver weight was 25.2 ± 0.71 while in age-matched treated SHR at the end of 3-week hemin protocol (n=7) it was 27.3 ± 2.2 ($p>0.05$). There was also no significant difference regarding body weight/kidney weight between hemin-treated SHR (98.3 ± 2.3) and the age-matched untreated 15 week old SHR (96.5 ± 3.5).

Age-matched 15 week old untreated SHR (n=7) and the treated SHR at the end of the 3-week hemin protocol (n=7) had comparable normal levels of serum urea (14.3 ± 2.2 vs. 15.7 ± 1.9 mgN/dl) and creatinine (0.9 ± 0.03 vs. 1.0 ± 0.06 mg/dl).

Normal serum urea and creatinine levels are 10-20 mgN/dl and 0.7-1.4 mg/dl, respectively.

Table 4.2. Body weights (gram) changes by the end of 3-week hemin or hydralazine treatment (n=20 per group)

Age (weeks)	SD Control	SD-hemin	SHR-control*	SHR-hemin*	SHR-vehicle*	SHR-hydralazine*
11	338.2 ± 2.2	343.2 ± 5.2	292.4 ± 4.8	296.7 ± 3.9	288.4 ± 4.3	278.4 ± 3.3
12	354.3 ± 3.4	356.4 ± 4.7	299.2 ± 4.6	300.4 ± 3.8	291.2 ± 4.5	284.2 ± 3.2
13	374.5 ± 3.7	378.7 ± 4.6	308.3 ± 3.7	307.3 ± 3.4	312.3 ± 4.6	307.3 ± 2.5
14	398.2 ± 4.3	397.7 ± 4.4	316.2 ± 4.5	314.7 ± 3.7	325.2 ± 4.8	321.2 ± 5.5
15	416.2 ± 4.5	417.5 ± 5.1	325.6 ± 3.5	322.4 ± 4.3	333.6 ± 5.1	329.6 ± 5.3

(*$p<0.05$ vs. SD rats)

4.3 Effect of hemin protocol on ICP of adult SHR and its underlying mechanisms

Male 10 week old SHR and SD rats were included in this study. Two weeks after animal acclimatization, hemin was administered intraperitoneally at a dose of 15 mg/kg/day for 21 consecutive days (hemin protocol) to 12 week-old SHR (n=14) and age-matched SD (n=14) rats. Control SHR (n=14) and control SD rats (n=14) were left untreated. Hydralazine (45 mg/kg/d) was given orally to another group of 12 week-old SHR for 21 days (n=6). Both systolic BP and mean arterial BP (MAP) were derived from tail-cuff BP measurement records. These measurements were conducted after acclimatization, two days prior to the start of the hemin or hydralazine therapy and successively on a daily basis for the entire 21-day period. Intracavernous pressure response was assessed in all animals. Protein expression levels using Western blot analysis were determined in penile tissues.

4.3.1 Normalization of BP in adult SHR after 3 week hemin protocol

The 15 week control SHR and SD rats weighed 323.5 ± 4.4 (n=14) vs. 417.2 ± 6.2 g (n=14), respectively, while the hydralazine-treated SHR weighed 337.4 ± 3.4 g. Hemin-treated SHR and their age-matched SD rats weighed 329.5 ± 5.4 (n=14) vs. 404.2 ± 2.6 (n=14) g, respectively. The 3-week hemin protocol significantly lowered BP of adult SHR from 168 ± 3.4 to 123 ± 1.2 mmHg (n=14, $p<0.001$) but had no effect on age-matched SD rats (119 ± 0.9 vs. 120 ± 0.2 mmHg, n=14). Similarly, after 3 weeks of oral hydralazine therapy, BP of SHR significantly decreased from 198.8 ± 2.2 to 129 ± 1.9 mmHg (n=6, $p<0.001$) (Fig. 4.15).

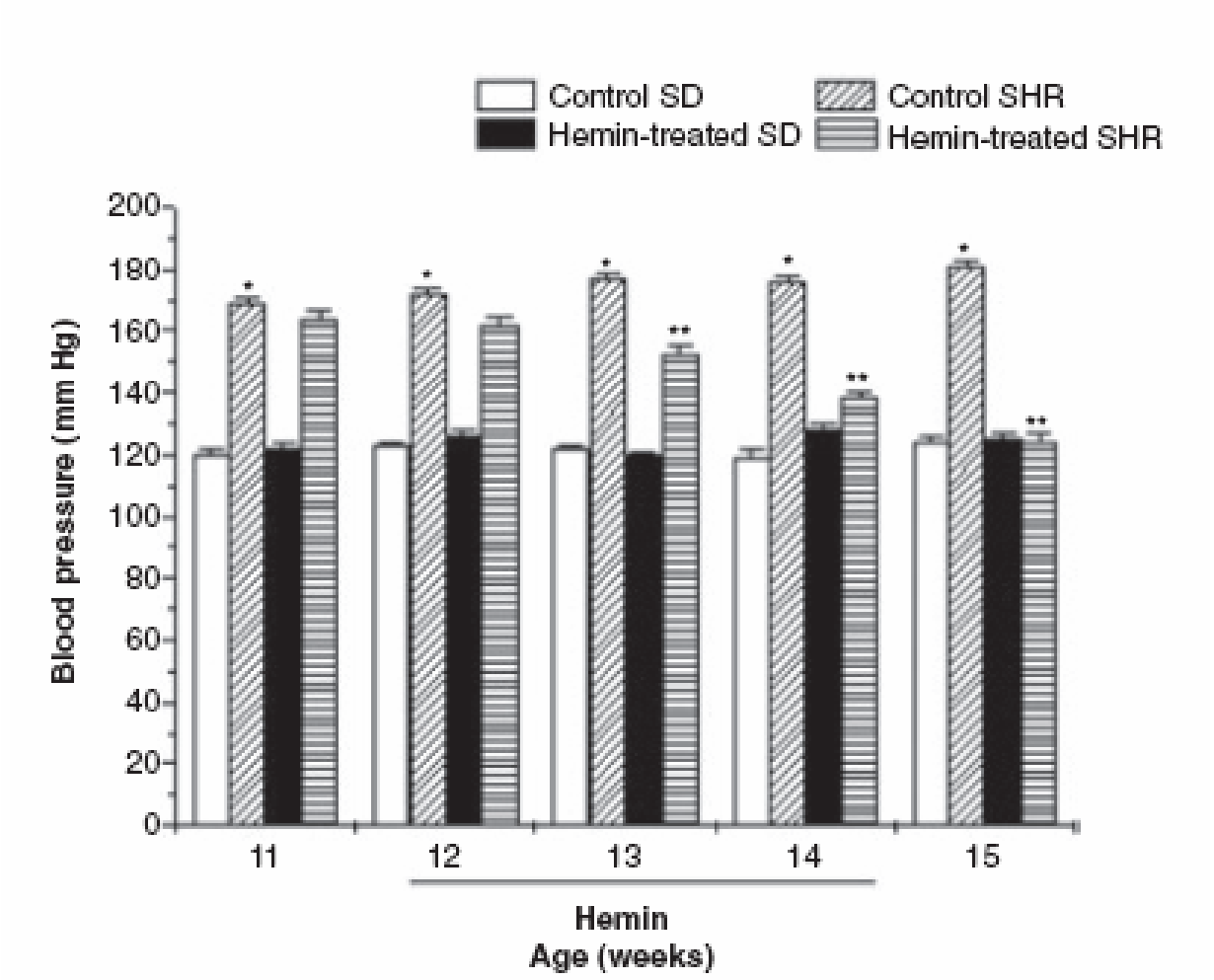

Figure 4.15 BP changes in experimental rats. Systolic BP was significantly higher in control SHR than in age-matched control SD rats (*P < 0.05). Three weeks after hemin therapy, systolic BP of SHR significantly decreased to normotensive BP values (**p < 0.05, hemin-treated SHR vs. control SHR).

4.3.2 ICP responses in control rats

Typical ICP responses elicited by electrical stimulations (6 V, 10 Hz, 1 ms, 1 minute) of the CN of control SD and SHR are displayed in Fig. 4.15A. In both SD and SHR rats, electrical stimulation induced a rapid increase in ICP associated with visible tumescence of the penis. This ICP increase was maintained as long as the stimulation lasted (plateau phase). After the end of the 1-minute stimulation period, ICP returned to its pre-stimulation level. The ICP during the plateau phase was significantly lower in SHR than in age-matched SD rats. MAP was significantly higher in anesthetized SHR (n=7) compared with anesthetized SD rats (n=7) (170 ± 1.0 vs. 106 ± 1.7 mmHg, $p < 0.05$). The ICP/MAP ratio increased in parallel with the frequency of CN electrical stimulation both in SHR and SD rats (Fig. 4.16B). However, the magnitude of the ICP responses was reduced drastically in SHR compared with SD rats at all stimulation frequencies ($p < 0.01$**;** Fig. 4.165B).

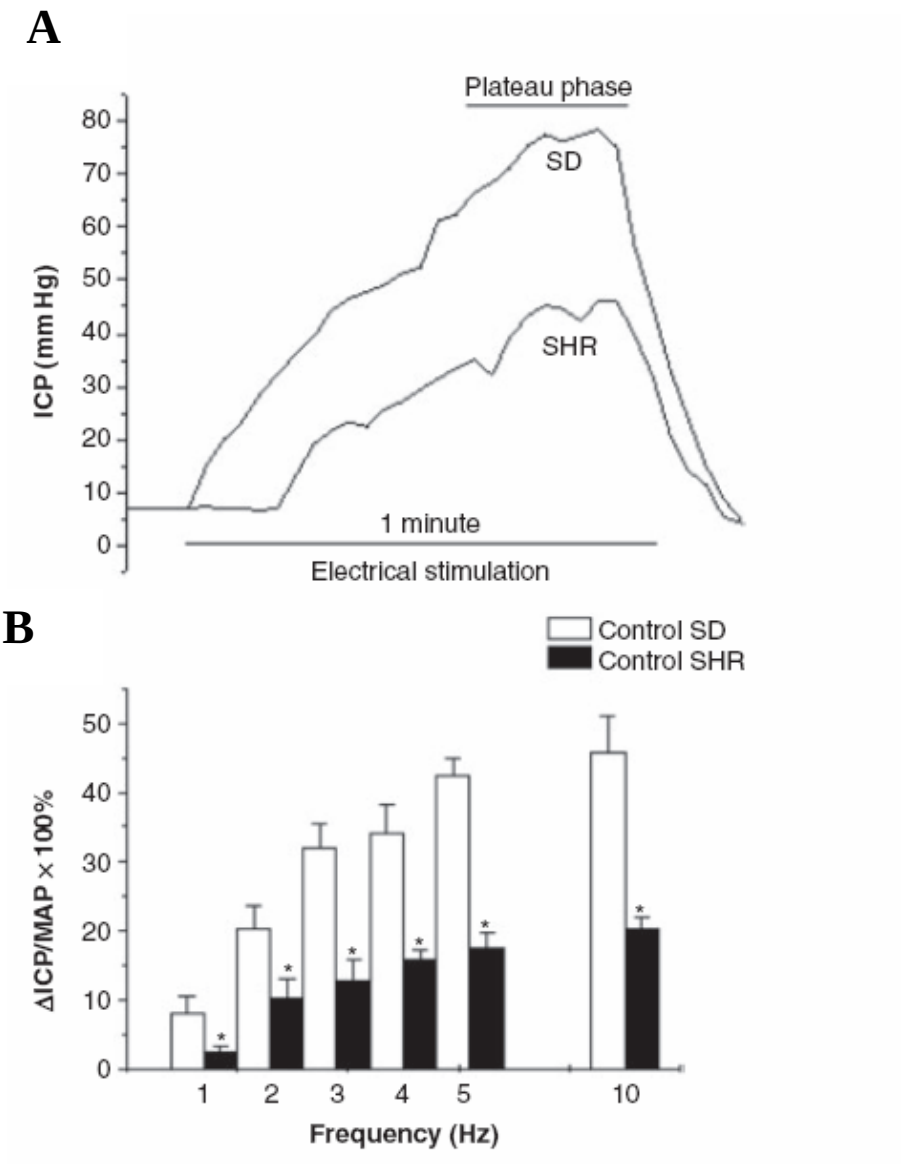

Figure 4.16 Effect of electrical stimulation of cavernous nerve (CN) in untreated control rats. A) Representative digitalized tracing of original recording of ICP when stimulating the CN (6 V, 10 Hz, 1 millisecond, 1 minute) of SHR and age-matched SD rats. MAP was significantly higher in anesthetized SHR (n = 7) compared with anesthetized SD rats (n = 7) (168 ± 1.0 vs. 106 ± 1.7 mm Hg, $p < 0.05$). B) Effect of CN stimulation at increasing frequencies on ICP of SHR and SD rats. Results are expressed as the ratio ΔICP (mm Hg)/MAP (mm Hg) ×100 during the plateau phase (*$p < 0.05$, SHR vs. SD rats).

4.3.3 ICP responses in hemin-treated and hydralazine-treated rats

Similar ICP responses were observed in hemin-treated SD rats (n=7) and treated SHR (n=7) with no significant difference (Fig. 4.17A). However, ICP responses in hydralazine-treated SHR were significantly lower than observed in the former 2 groups (Fig 4.17). MAP of hemin-treated SD rats, hemin-treated SHR and hydralazine-treated SHR were not different during cavernous nerve stimulation (102 ± 2.2, 98 ± 2.8, 104 ± 3.12 mmHg, respectively). The ICP/MAP ratio increased in parallel with the frequency of CN electrical stimulation both in hemin-treated SHR and SD rats with similar magnitude, but not in hydralazine-treated SHR (Fig. 4.17B). ICP responses increased significantly in hemin-treated SHR in comparison with that of control SHR and hydralazine-treated SHR (Fig. 4.17B) ($p<0.05$).

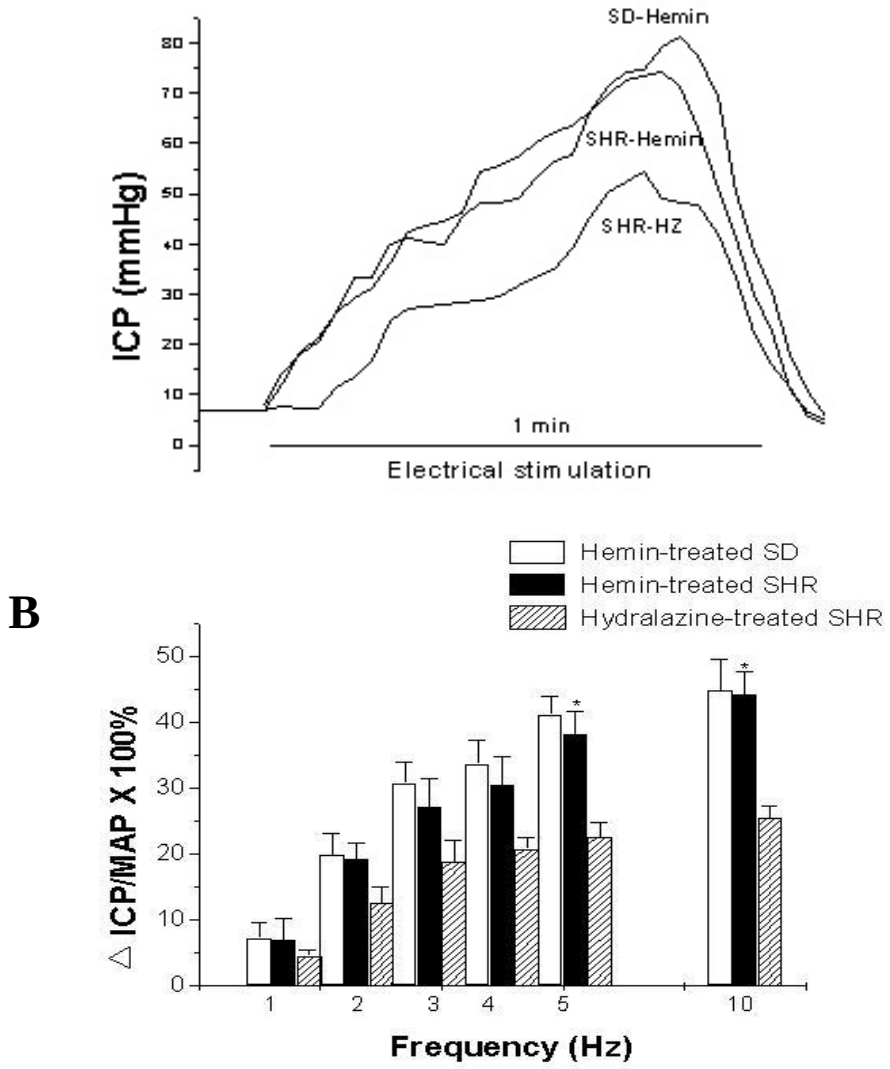

Figure 4.17 Effect of electrical stimulation of cavernous nerve (CN) in hemin- or hydralazine-treated rats. A) Representative digitalized tracing of original recording of ICP when stimulating the CN (6 V, 10 Hz, 1 millisecond, 1 minute) in SHR and age-matched SD rats. MAP of hemin-treated SD rats, hemin-treated SHR, and hydralazine-treated SHR were not different during CN stimulation (102 ± 2.2, 98 ± 2.8, 104 ± 3.12 mm Hg, respectively). B) Effect of CN stimulation at increasing stimulation frequencies on ICP of SHR and SD rats. Results are expressed as the ratio ΔICP (mm Hg)/MAP (mm Hg) ×100 during the plateau phase (*$p < 0.05$, hemin-treated SHR vs. hydralazine-treated SHR). HZ=hydralazine.

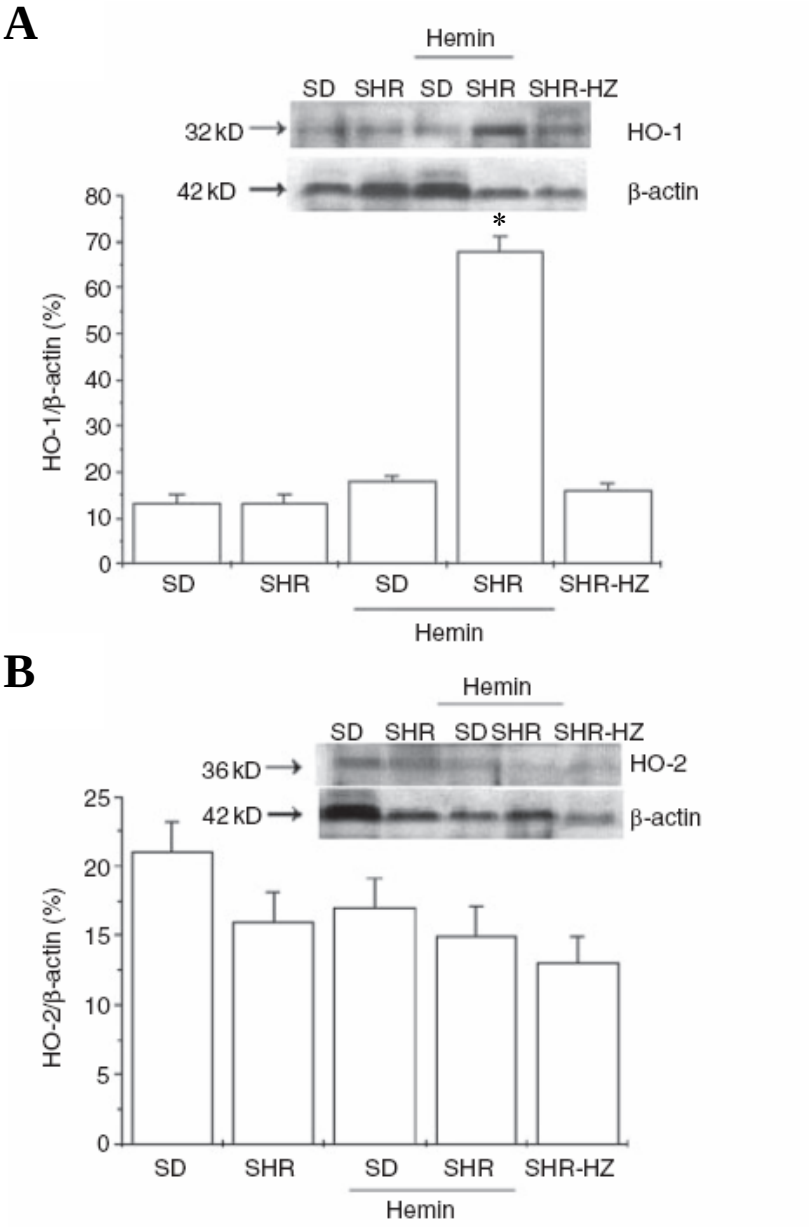

Figure 4.18 Expressions of HO-1 and HO-2 in the penile tissue of adult SHR and SD rats. A) Representative Western blot and relative abundance expression levels of HO-1. *p< 0.05 vs. all other groups; n = 7 for each group except n = 6 for hydralazine-treated SHR. B) Representative Western blot and relative abundance expression levels of HO-2. n = 7 for each group except n = 6 for hydralazine-treated SHR. HZ = hydralazine.

4.3.4 Upregulation of the expression of HO-1 and sGC, but not HO-2, by hemin protocol

Hemin protocol significantly increased HO-1 expression in adult SHR but not in SD rats (Fig. 4.18A). No significant difference was detected in basal expression of HO-2 in the cavernous tissue of control SD and SHR rats. Furthermore, HO-2 protein expression remained unaltered following the application of hemin protocol (Fig 4.18B). Hydralazine therapy failed to increase either HO-1 or HO-2 in the cavernous tissue of SHR.

No significant difference was detected in the basal expression of sGC in the cavernous tissue of control SD and SHR rats (Fig. 4.19). Chronic hemin application significantly increased the expression level of sGC in SHR, but not in SD rats (Fig. 4.19).

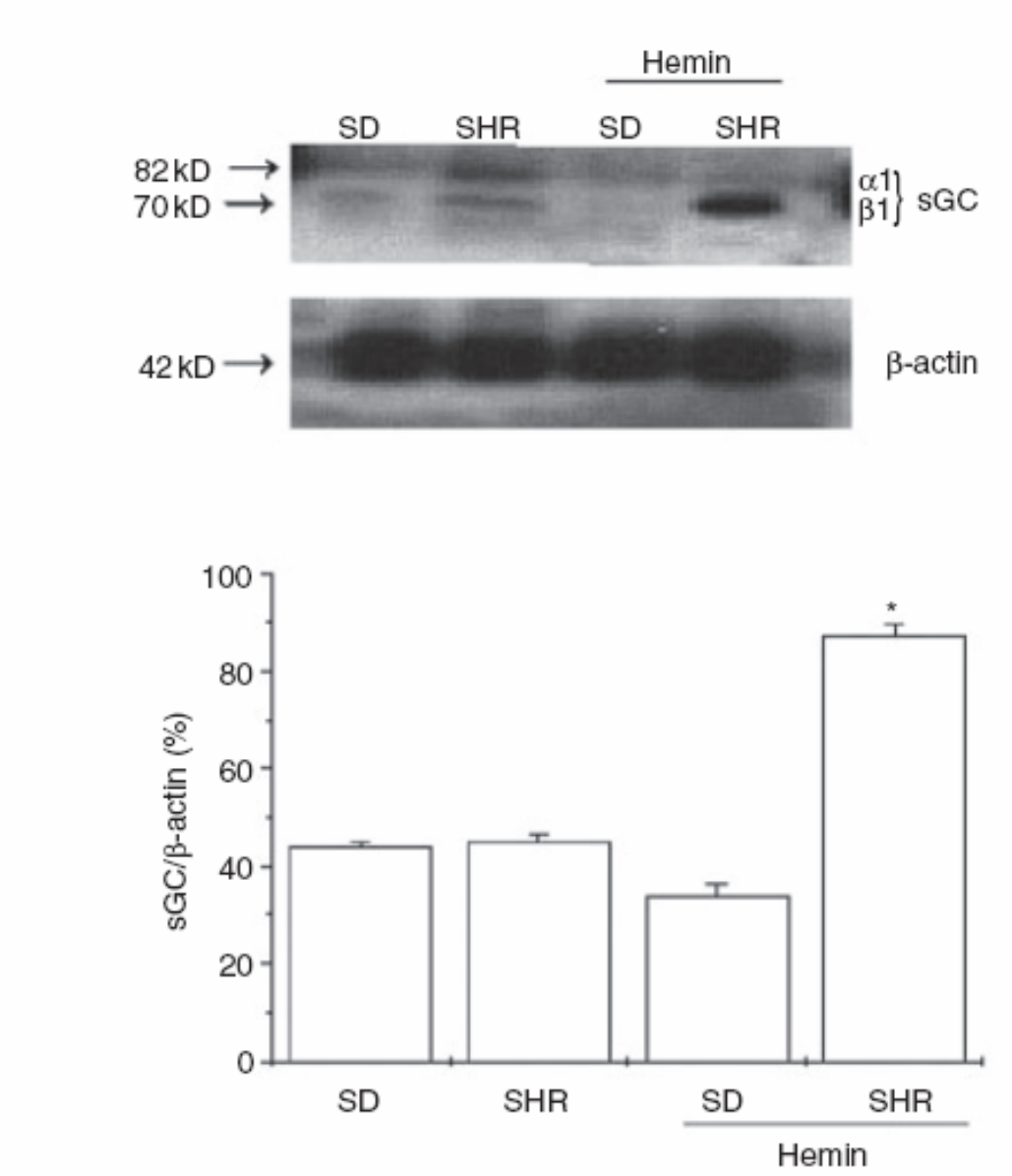

Figure 4.19 Expression of sGC protein in the penile tissue of adult SHR and SD rats. Representative Western blot is shown on the top, and the mean relative abundance levels of expressed sGC proteins are shown at the bottom. *$p < 0.05$ vs. all other groups; $n = 7$ for each group.

4.3.5 Downregulation of PDE5 by hemin protocol

PDE5 is the most abundant phosphodiesterase in the cavernous tissue. Basal expression levels of PDE5 in SHR were significantly higher than that in age-matched SD rats (Fig. 4.20). Chronic hemin treatment markedly decreased the expression of PDE5 in SHR in comparison to the SD rats (Fig. 4.20).

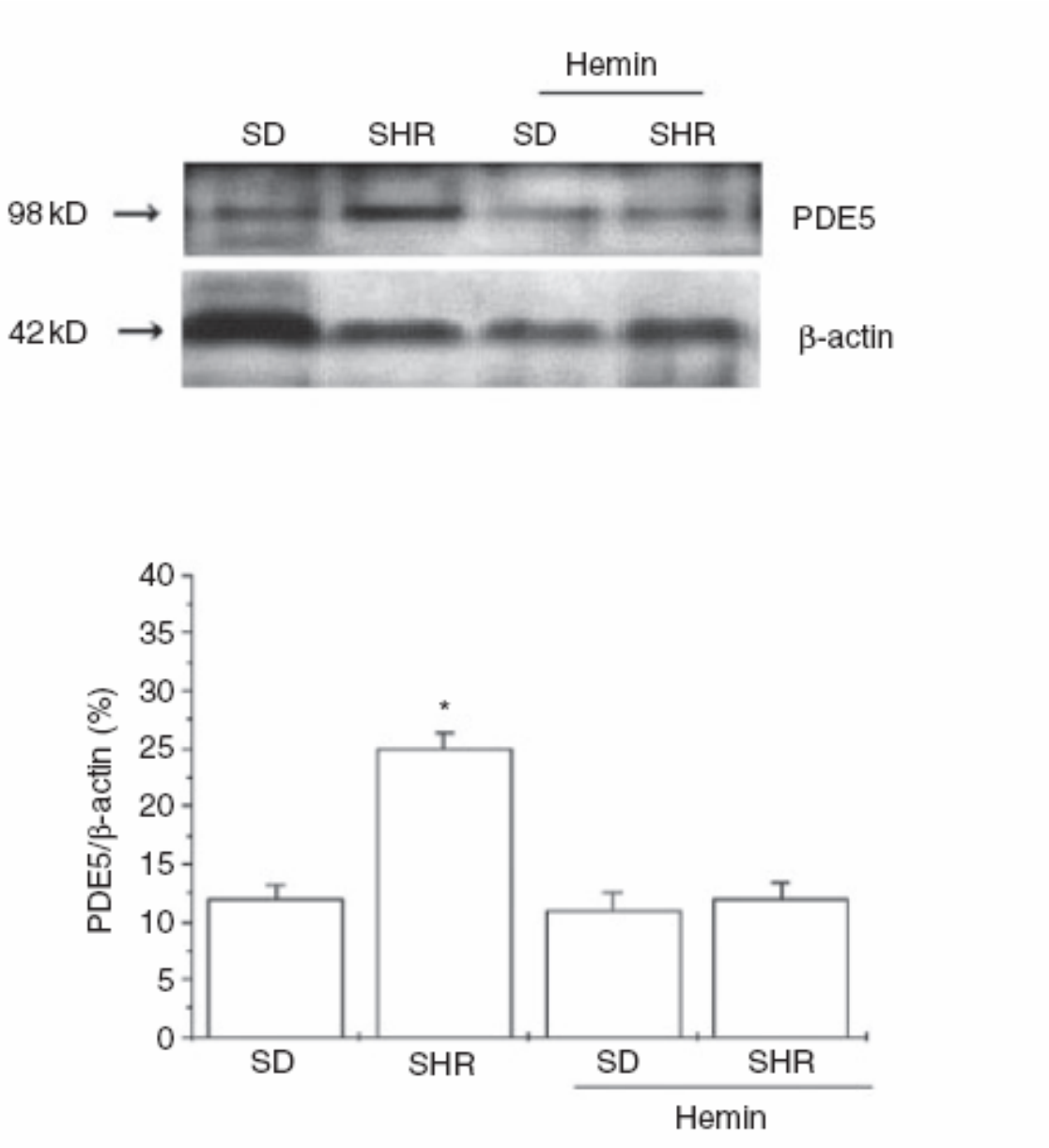

Figure 4.20 Expression of PDE5 protein in the penile tissue of adult SHR and SD rats. Western blot results reveal significantly elevated PDE5 expression in the penile tissue of adult SHR that was suppressed by the hemin protocol. *$p < 0.05$ vs. all other groups; $n = 7$ for each group.

5. Discussion

5.1 Metabolism of heme and its derivatives

Early reports since the 1960s have indicated that heme, besides being synthesized in mitochondria in every cell type, is also transported from plasma into erythroid and non-erythroid cells such as hepatocytes, non-erythroid progenitors muscle cells and fibroblasts) (Smith and Morgan, 1964). Heme, as the most bioavailable dietary form of iron, is also transported into enterocytes. The level of intracellular heme in a given cell is regulated by *de novo* heme biosynthesis, utilization of heme being released by the microsomal enzymes HOs, as well as by the amount of free heme imported in or exported out of the cells (Ponka 1999). This regulation is quite critical as heme is considered an important biological product, being part of vital hemoproteins that execute basic cell functions as well as being toxic at relatively high intracellular levels (Tsiftsoglou et al., 2006). The latter is important because heme promotes lipid peroxidation and tissue damage via radical oxygen species (ROS) (Balla et al., 1993).

Smith and Morgan (1964) indicated that heme released from hemoglobin in plasma, following red blood cell degradation in the spleen, is transported into liver cells via hemopexin, a carrier plasma protein (Tsiftsoglou et al., 2006). Hemopexin forms stable complexes with heme that are internalized inside the cells. Hemopexin returns intact to the extracellular space just after releasing exogenous heme into intracellular cytosolic compartment for reutilization. This carrier-mediated import of

heme via hemopexin is a saturation-, time-, and energy-dependent as well as tissue-specific process. Iron released from heme by microsomal HO is then stored into ferritin. A human plasma membrane transporter for heme has been detected in the intestine (Worthington et al., 2001). Most recently, a new heme carrier protein-1 (HCP1) has been detected in duodenal intestinal cells that share homology to the bacterial metaltetracycline transporters. This protein is highly expressed in duodenum, is regulated by hypoxia and iron, and mediates dietary heme (Shayeghi et al., 2005). Heme may be also transported by facilitated passive diffusion due to its hydrophobic characteristics and ability to interact with phospholipid membrane components at the cell surface (Light and Olson 1990).

Heme-binding proteins (HeBPs) acting like heme receptors were detected in murine erythroleukemia (MEL) cells (Galbraith et al., 1985) and human erythroleukemia (K-562) cells as well (Majuri, 1989; Tsiftsoglou et al., 1992). Moreover, a number of mammalian HeBPs and/or tetrapyrrole-binding proteins were isolated from rabbit serum as well as from rat liver cells (Vincent and Muller-Eberhard, 1985; Iwahara et al., 1995; Taketani et al., 1998; Taketani, 2005). These findings indicate that heme is transported via hemopexin and/or HCP1 (energy-dependent process) and may be via facilitated passive diffusion. Soon after, heme enters the intracellular space and interacts with the cytosolic proteins (cytosolic hemebinding proteins; CBP), like the p23 kDa heme-binding protein found in liver (Iwahara et al., 1995). Unfortunately, no detailed analysis of these heme import systems has been completed thus far, and current knowledge on how heme enters

proerythroid and non-erythroid cells at the molecular level still remains inadequate (Tsiftsoglou et al., 2006).

Circulatory heme levels have not been previously reported in hypertensive patients or experimental animals. In the present study, we firstly examined the accuracy of a spectrophotometric assay for circulatory heme levels *in vitro*. A near linear relationship between the known hemin concentrations and the absorbance difference of reduced pyridine hemochrome was established. Addition of the lysinate residue to hemin appeared not to alter the heme level *in vitro*. The standard calibration curve for HLL is similar to that of hemin, albeit less linear especially between 10-30 micromol/L. The calibration range for hemin or HLL (1-30 micromol/L) was chosen based on previous reports that the normal circulatory heme level was either undetectable or less than 1 micromol/L (Yachie et al., 1999). With the establishment of the sensitive heme assay, the serum levels of heme and BP changes in hypertensive SHR and normotensive SD rats with or without hemin/HLL treatments were further correlated. The results showed that, although BP levels were significantly different between SHR and SD rats, serum heme levels were similar, i.e. negligible, in these two strains of rats.

Immediately after hemin/HLL injection, serum heme level increased significantly. This elevated serum heme level remained unchanged throughout the injection period, but gradually declined back to the basal level after the termination of the hemin/HLL injections. This profile of circulatory heme levels may be related to metabolic characteristics of heme. Slow catabolism of heme after injection of hemin or HLL could be limited by the availability of HO expression level and HO activity.

Excess heme will remain in circulation until more HO-1 proteins are induced and total HO activity is increased to counteract the increase in heme level. It is speculated that during hemin/HLL injection, heme was absorbed from the injection site into the circulation. Further distribution of circulatory heme to various organs and tissues induces HO-1 expression locally, especially in vascular tissues. Upregulated HO expression and activity produces more CO and bilirubin. Vasorelaxation, reduced vascular inflammation, and reversal of hypertension-related vascular remodeling may all occur subsequently, contributing to the BP-lowering effect of hemin/HLL injections.

HLL has been used to decrease BP in SHR, owing to the increased stability and membrane solubility of hemin solution after addition of lysine residue (Martasek et al., 1991). However, in the present study, HLL did not prove superior to hemin regarding its BP lowering effect. A reasonable explanation for this is that both hemin and HLL injection routines yield similar patterns and extent of the elevation of serum heme levels.

To examine whether serum heme levels can differ according to age or species, hemin or HLL was injected in older (>20 weeks) SHR and age-matched SD rats for 5 days. BP levels remained unchanged in these animals during and after hemin/HLL injections, which were similar to previous reports demonstrating no effect of hemin injection on BP of old SHR or SD rats (Ndisang et al., 2003). Serum heme levels in these animals during hemin/HLL injections were significantly increased compared with the pre-injection levels. Cessation of hemin/HLL injections resulted in a gradual decline of serum heme levels reaching baseline levels approximately 2 weeks later

with no change in BP of all rats. However, serum heme levels were not different between SHR and age-matched SD rats during and after injections with either hemin or HLL.

Several issues related to this heme assay should be mentioned. First, as the pyridine spectrophotometric reagent forms nitrogen bonds with heme, differentiation between heme (Fe^{+2}) and hemin (Fe^{+3}) cannot be made. Therefore, the serum heme level measured in this study is the summation of intrinsic heme present in the circulation and the exogenously administered hemin or HLL. Since the baseline endogenous heme level is very low and the termination of hemin/HLL injection resulted in decrease in serum heme to baseline levels, it may be concluded that any significant increase in serum heme levels is due to the hemin/HLL injected. Second, only ~ 85% of heme present in the serum sample could be detected using this classical spectrophotmetric method (Maines 1999). This underestimation should be kept in mind when interpreting the measured serum heme data. Though using fluorescent or high performance liquid chromatography (HPLC) (Maines 1999) methods could have presented a more accurate estimate of heme levels, both methods are more suitable in detection of tissue heme rather than serum heme. Also, the fluorescent method necessitates the introduction of a fluorescent dye into the circulation, which might carry a risk of toxicity. Recently, Lombardo et al (2005) reported a new method using chloroform to extract hemin added to biological extracts, followed by spectrophotometric assay of chloroform absorbance. The authors report a 15-30 fold higher sensitivity of their method in comparison to the pyridine hemochromogen assay. However, their method has only been employed for

the determination of hemin added artificially to a solution or a biological extract with no *in vivo* results reported.

5.2 Effects of 13-day hemin treatment on BP of adult SHR

The effects of a short-term (4 days) hemin injection (i.p.) regimen on hypertension development in SHR aged 8 or 20 weeks old have been previously studied (Ndisang et al., 2003). This hemin injection regimen lowered BP of 8-week SHR, but not that of 20-week SHR. In the present study, a 5-day hemin therapy also failed to lower high BP in SHR older than 20 weeks. Around 8 weeks of age BP is increasing but not fully established in SHR. At this age range, a 4-day hemin injection period suffices to bring down high BP (Tenhunene et al., 1987). When SHR reach an age equal to or older than 20 weeks, hypertension is fully developed and a 4-day hemin therapy fails to lower high BP (Ndisang et al., 2002).

In the present study, the effect of 13-day hemin injection (i.p.) therapy on BP of 12 week old SHR and age-matched SD rats was examined. Hemin treatment significantly decreased systolic and mean BP of SHR while having no effect on BP in SD rats. Interestingly, the drop in BP of 12 week old SHR began after the fifth day of injection and continued until the end of 13-day injections. These results suggest that the variance in the BP lowering efficacy of hemin therapy (i.p.) in SHR at different ages largely relies on the intensity and duration of hemin injections. With a prolonged daily hemin injection therapy, such as the one for more than 5 days, established high BP in adult SHR can be lowered. Following this logic, it can be

predicted that an even longer hemin injection period beyond 13 days would normalize BP and have long-lasting effect in SHR with established hypertension.

5.3 Effect of a 3-week hemin protocol on BP of adult SHR

Different pharmacological and genetic approaches have been utilized to upregulate HO-1 with the ultimate goal of sustained normalization of BP in SHR (Ndisang et al., 2004). However, these therapeutic modalities only modestly lowered high BP in young SHR for a short period of time. Once hypertension is fully established in SHR, upregulating HO failed to lower BP (Ndisang et al., 2004). More importantly, a long-lasting normalization of high BP in the absence of anti-hypertensive interventions has not been realized clinically or experimentally. In responding to these challenges, a 9-month normalization of BP in adult SHR after a 3-week hemin treatment protocol is reported in this thesis.

Controlled subcutaneous drug release is the preferred means of drug delivery over extended periods of time (Karl-Heinz et al., 2001). It avoids common drawbacks of the intraperitoneal injection, namely, drug absorption to the portal circulation and peritoneal adhesions. Additionally, controlled subcutaneous release allows an evenly-distributed hourly drug discharge instead of a single-shot bolus delivery (Karl-Heinz et al., 2001). In the present study, adult SHR were treated with hemin via subcutaneously implanted miniosmotic pumps for 3 weeks. The choice of the 3-week hemin protocol was based on previous findings that a 13-day hemin treatment (i.p.) significantly lowered BP in 12 week-old SHR (Shamloul and Wang 2005). Furthermore, our unpublished observations indicate that a 3-week i.p. injection of

hemin into adult SHR lowered the high BP to a normotensive level (Wang et al., 2005). It was found that BP of 12 week old SHR was normalized 2 weeks after the implantation of subcutaneous hemin-releasing pumps whereas hemin treatment via i.p. injection would not normalize BP in adult SHR until treatment was prolonged to 3 weeks (Wang et al., 2005). In order to confirm whether the anti-hypertensive effect of hemin had been stabilized, we continued treatment of SHR with hemin pump for one more week. During the 3rd week of hemin protocol, there was no further change in BP level in treated SHR. Restoration of BP in adult SHR with established hypertension to normotensive level by HO-1 inducers has not been reported to date and clearly this challenge has been met by these results.

Responsible genes for hypertension development in SHR have not been determined yet (Lerman et al., 2005). Numerous studies have shown that WKY, initially characterized as a genetic control of SHR, has many genetic traits different from SHR that may not relate to BP regulation at all (Yamori 1999; Fortepiani et al., 2003; Simpson et al., 1994). Since both WKY and SD rats are normotensive, we chose SD rats as the normotensive control in the present study.

5.4 Direct and indirect methods for BP measurements in experimental animals

Techniques for measuring BP in experimental animals can be divided into direct methods and indirect methods. Direct BP methods use radiotelemetric devices or indwelling catheters, implanted inside the animal's body and connected to a major

blood vessel to record BP. On the other hand, indirect BP methods are non-invasive techniques used to measure BP with no surgery needed.

In animals, the most commonly used indirect method for monitoring BP is the cuff technique in which BP is measured in a tail or limb by determining the cuff pressure at which changes in blood flow occur during occlusion or release of the cuff. The tail-cuff technique used to measure BP is considered to have 4 main advantages (Van Vliet et al., 2000; Meneton et al., 2000; Krege et al., 1995): (1) it is noninvasive and does not require surgery; (2) it can be used to obtain repeated measurements of systolic BP in conscious animals during studies of short or long duration; (3) it requires less expensive equipment than some direct methods (eg, telemetry) and can also be less expensive to operate; and (4) it can be used to screen for systolic hypertension or substantial differences in systolic BP among large numbers of animals. However, recent recommendations of measuring BP in experimental animals pointed out a few limitations of the tail-cuff method. First, indirect methods only measure BP in a very small sample of cardiac cycles. Second, these methods impose significant stress on the animal that disturbs multiple aspects of the cardiovascular system. Kurtz et al (2005) recommended the use of radiotelemetry to allow for continuous, direct measurements of BP without the need for restraint or the use of tethering devices.

It is generally advised that investigators first establish the physical accuracy of an indirect method by calibration against a mercury column and by comparing indirect pressure measurements to simultaneously obtained direct measurements of arterial pressure (Hassler et al., 1979). The assessment of an indirect method should

include attention to technical details, including cuff size (Bunag 1983). Comparisons to a direct method should be performed using appropriate techniques of agreement analysis rather than simple correlation/regression analyses (Jamieson et al., 1997). It is usually recommended that the animals be exposed to the measurement procedures every day for 7 to 14 days before the beginning of an experiment. Thus, in our study, and to reduce the effect of stress on animals, BP of all animals was measured daily for 2 weeks using tail-cuff prior to the beginning of actual experiments. Investigators have also offered a variety of tips to reduce stress and improve measurement reliability including placement of a dark cover over the animals, use of a single technician to conduct the measurements at the same time each day, and the use of clean equipment free from foreign scent and blood odor (Meneton et al., 2000; Bunag 1983). All these tips were adapted into our BP measurement protocol.

It has been recommended that results obtained by the tail-cuff technique be verified by direct BP measurements (Bunag 1983). In our study no significant difference was observed in systolic BP measurements between direct and indirect methods (Fig. 4.8). In both techniques SHR treated with hemin had significantly lower BP in comparison to untreated SHR, confirming hemin protocol hypotensive effects. The lack of significant differences regarding systolic BP measurements between tail-cuff and radiotelemetry in untreated SHR is in disagreement with previous reports. Bazil et al (1993) reported significantly higher systolic BP values obtained from SHR using tail-cuff than those obtained through radiotelemetry, 210 ± 4 mmHg vs 168 ± 2 mmHg, respectively. The small number of animals implanted with radiotelemetric devices in our study may be the reason for this disagreement.

Also, the possibility of some degree of restlessness encountered by the animals during radiotelemetric recording may be considered another contributing factor. Therefore, the comparison between radiotelemetry and tail-cuff BP measurements in our study should be interpreted cautiously.

5.5 The interaction between HO/CO and NO/NOS systems

Vascular HO-1 expression levels in mesenteric arteries were significantly higher in untreated adult SHR than age-matched SD rats, the same case as for iNOS expression levels (Cheng et al., 2004). An impairment of endothelium-dependent relaxation has been observed in different vessels from SHR (Marin and Rodriguez-Martinez 1997). This suggests that endothelial function might be impaired in hypertension. However, numerous studies have demonstrated that NO synthesis can be increased in SHR, probably as a counter-regulatory mechanism activated to compensate for the increase in BP (Chou et al., 1998). Therefore, the precise role of NO in hypertension is not clear, although a role of HO-1 in hypertension has been implied (Johnson et al., 1997). Moreover, some investigators have reported up-regulation of HO-1 expression in hypertension and have suggested that the HO-1/CO system contributes to BP regulation in SHR (Chou et al., 1998).

Cheng et al (2004) investigated the expression levels of HO-1 and iNOS in SHR at different ages. They reported that BP may play an important role in the modulation of HO-1 and iNOS expression in aorta during the development of SHR (Cheng et al., 2004). Their results also suggest that the increase of HO-1 and iNOS expression may exert an important compensatory effect on the elevation of BP. HO-1

was induced at the early stage around 8 weeks and progressively maintained during the whole experimental period, whereas, iNOS was expressed at a later stage (12 to 16 weeks). For the other animals, the BP was significantly increased after administration of zinc protoporphyrin (HO inhibitor) for 7 consecutive days from age 7 weeks. Chronic blockade of iNOS activity by aminoguanidine from 5 weeks to 16 weeks of age resulted in significant up-regulation of HO-1 expression, but a pressor effect was not found. These results suggest that the HO-1/CO system may compensate for the dysfunction of the iNOS/NO system and play a major role in the regulation of BP during the development of hypertension in SHR (Cheng et al., 2004).

Despite the increase in HO-1 and iNOS expression, arterial BP still increased in SHR. One possible explanation is that the vascular superoxide anion level in SHR increased in an age-dependent manner in concordance with the development of elevated BP (Zalba et al., 2000). The superoxide anion can scavenge NO to form peroxynitrite, which leads to a decrease in the availability of NO or to injury of the vascular bed, or both. The other possible explanation is that elements of the NOS/NO-sGC/cGMP system or the HO/CO-sGC/cGMP system were impaired (Ndisang and Wang 2003). Therefore, despite the observed compensatory increase in HO-1 and iNOS expression, BP still increased during the development of hypertension in SHR.

The mechanism responsible for upregulation of HO-1 and iNOS expression during the progression of hypertension in SHR is still not clear. In animal studies, arterial superoxide anion levels were increased in several hypertensive models

including SHR (Kerr et al., 1999). Although the source of superoxide anion is uncertain, several observations suggest that the reduced nicotinamide adenine dinucleotide (NADH)/ reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system accounts for the majority of superoxide anion generation in the vessel wall (Mohazzab et al., 1994). These data suggest that the superoxide anion may play an initial and critical factor in the induction of HO-1 and iNOS expression. Thus, the possibility that an increase in superoxide anions in SHR may be secondary to an increase in BP cannot be excluded and requires further study (Cheng et al., 2004). It is apparent that the HO/CO system can take over and become a major modulator for the regulation of BP when the iNOS/NO system is suppressed (Cheng et al., 2004). The specific mechanism for expression of HO-1 and iNOS in SHR remains to be clarified and requires further study. This may represent an adaptive reaction of vascular tissues to counter high vascular contractility with hypertension in adult SHR.

The cross-talk between the HO/CO system and NOS/NO system is well known (Foresti et al., 2002). For instance, exposure of endothelial cells to CO augmented the release of NO (Thom et al., 2000). However, in the present study no expressional changes were detected in NOS in vascular tissues from hemin-treated SHR. Therefore, normalization of BP in these animals cannot be attributed to the altered NOS/NO system.

5.6 Mechanisms of HO-1 upregulation-mediated antihypertensive effect

HO-1 expression and total HO activity were significantly upregulated in hemin-treated SHR, but not so much in hemin-treated SD rats. Increased total HO activity would generate more CO and the latter relaxes vascular tissues and decreases peripheral resistance. Vascular production of bilirubin and biliverdin will also be increased with HO-1 upregulation. As potent endogenous antioxidants and anti-inflammatory factors (Kaur et al., 2003), bilirubin and biliverdin suppress the oxidative stress that normally accompanies hypertension (Wu and Juurlink 2002). Iron, an important constituent of the heme moiety, is shuttled to intracellular stores such as ferritin (Stenqvist 2000). The removal of free iron by ferritin abolishes iron(II)-induced oxidation (Ferris et al., 1999). Additionally, iron enhances ferritin synthesis and the latter also possesses antioxidant and anti-inflammatory properties (Ferris et al., 1999). All these beneficial acts may have contributed to normalization of BP in hemin-treated SHR.

Amongst the 11 different known PDEs, only PDE5, PDE6 and PDE9 are specific for hydrolyzing cGMP (Wallis et al., 1999) and the presence of PDE5 in the mesenteric artery has been reported (Schipper 2000). Although the mechanism for the downregulation of PDE5 by hemin treatment, as reported in the present study, are unclear, this effect together with increased sGC expression in mesenteric arteries both contribute to increased vascular cGMP content in hemin-treated adult SHR.

5.7 Mechanisms underlying long-lasting antihypertensive effects of hemin protocol in SHR

In many types of experimental hypertension, including SHR, eutrophic vascular remodeling is characteristically found in resistance arteries as decreased outer diameter and lumen size, unchanged cross-sectional area (CSA) of the media, and a greater media-lumen ratio (Baumbach and Heistad 1989). Eutrophic vascular remodeling also manifested itself in mild essential hypertensive patients (Rosei et al., 1995). In our study, eutrophic vascular remodeling of adult SHR was reversed after the hemin protocol. The lumen diameter of small mesenteric arteries of SHR was increased and media/lumen ratio decreased without any change in the CSA (Table 1). These intriguing structural changes probably underlie the long-lasting normalization of BP observed in this study.

Basic and clinical research have shown that hypertension is clearly linked to inflammation (Nomura et al., 2002). Upregulation of genes related to inflammation, such as tumor necrosis factor-α, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1, were all observed in target organs and/or circulation of hypertensive animals and patients (Nomura et al., 2002). It has been suggested that angiotensin II (Ang-II), a potent endogenous vasoconstrictor, is responsible for the inflammatory changes occurring in hypertension and contributing to vascular remodeling through its effect on VEGF (Baumgartner and Isner 2001). There is emerging evidence that VEGF induces migration and activation of monocytes through induction of adhesion molecules or chemokines such as monocyte chemoattractant protein-1 (MCP-1) (Barleon et al., 1996). An overexpression of

VEGF in mesenteric arteries of adult SHR in comparison with that of normotensive SD rats is reported in this study. Normalization of the VEGF levels after hemin protocol was associated with significant structural changes in peripheral mesenteric arteries of SHR leading to reversal of hypertension-associated remodeling.

In 1993, Ku et al demonstrated that VEGF induces endothelium-dependent relaxation of isolated canine coronary arteries. VEGF preferentially dilates arterioles and venules without an effect on medium-sized arteries and veins (Laham et al., 2003). In the VIVA (VEGF in Ischemia for Vascular Angiogenesis) trial, intracoronary and intravenous infusions of recombinant human VEGF produced falls in systolic BP of up to 22% at the highest doses (Henry et al., 2003). It is postulated that this hypotensive effect of VEGF is related to enhancement of eNOS activity and expression levels in endothelial cells (Hood et al., 1998). However, contrary to these reports several other recent studies pointed that VEGF may be involved in the pathogenesis of hypertension. In an interesting study, Zhao et al (2004) demonstrated that VEGF is an essential mediator in Ang II–induced vascular inflammation and structural changes through its proinflammatory actions. Blocking of VEGF resulted in attenuation of vascular inflammation and remodeling in hypertension (Zhao et al., 2004). In another clinical study, Felmeden and colleagues (2003) demonstrated a positive correlation between hypertension and higher VEGF levels. These authors reported a significant reduction in the plasma VEGF levels following 6 months on intensive cardiovascular risk factor management. Nevertheless, the mechanism by which VEGF expression and activity are controlled in hypertension remains unclear.

HO-1 activity increases the generation of VEGF in vascular SMCs in both normoxic and hypoxic conditions (Dulak et al., 2002). Paradoxically, the present study shows that increased HO-1 activity led to a pronounced decrease in the expression of VEGF in mesenteric arteries of adult SHR at the end of the 3-week hemin protocol. These seemingly contradictory effects of HO-1 activity on VEGF expression can be reconciled by the hypothesis that the basal expression level of VEGF determines the effect of HO-1. Under normal or hypoxic conditions VEGF expression level is within the normal range and induction of HO-1 leads to elevation of its expression. In chronic hypertension, the basal level of VEGF is already significantly elevated and increased HO-1 activity then leads to a down-regulation of VEGF expression. The restoration of VEGF in SHR to a comparable level with that of normotensive SD rats led to a momentous reversal of eutrophic remodeling of mesenteric arteries (Table 1).

The hemin protocol reversed the vascular lumen size of SHR to dimensions comparable to that of normotensive rats at 15 and 55 weeks of age. Several studies have shown that 6-12 week chronic treatment of young SHR with antihypertensive agents such as angiotensin converting enzyme (ACE) inhibitors (Harrap et al., 1990) or angiotensin-1 (AT1) receptor antagonists (Lee et al., 1991) prevents BP from reaching fully hypertensive levels in adulthood. While these findings are interesting, however, their clinical implication is not of great magnitude. SHR differs from human hypertension in that SHRs reproducibly develop hypertension in young adulthood rather in middle age as in humans (Doggrell and Brown 1998). Also, despite the fact that certain groups of the population are at higher risk of hypertension

development, there appears to be no specific means to predict this disease during early adulthood years (Lauer et al., 1991).

Long-term antihypertensive pharmacological treatments started in adult SHR with ACE inhibitors (Harrap et al., 1990) or AT1 receptor antagonists (Oddie et al., 1993) reported no persistent antihypertensive effects following drug withdrawal. In a recent study, Paull and Widdop (2001) investigated the long-term effect of 3 different anti-hypertensives (perindopril, candesartan cilexetil, hydralazine) on BP of 18-20 weeks old SHR after 4 weeks of continuous treatment. Withdrawal of treatments resulted in rebound of BP to different degrees. Daily analysis showed that MAP returned to hypertensive levels within 4 days following withdrawal of hydralazine, 15 days after withdrawal of candesartan cilexetil, but after 8 weeks in SHR treated with perindopril. Other studies also reported similar long-lasting decreases of BP in adult SHR between 4-8 weeks after cessation of anti-hypertension treatments of adult SHR (Heller and Hellerova 1998). The most significant discovery of the present study is the long-lasting antihypertensive effect of the hemin protocol on adult SHR, which lasted for at least 9 months after the removal of hemin pumps.

The vascular perfusion method is the oldest method used for vascular morphology evaluation (Folkow et al., 1958). In this study, Folkow and colleagues examined the vascular response of resistance arteries to infused vasoconstrictor agonists. They reported that increased vascular reactivity in response to vasoconstrictor agents could be explained by an increased wall/lumen ratio that resulted in an encroachment of the vascular lumen (Folkow et al., 1958). Although this kind of approach provides information on the prevailing hemodynamics, it only

provides an indirect estimate of the average internal diameter of the perfused vasculature but no actual information on wall thickness. It also does not tell the investigator anything about which vessels are responding to the agonists (Bund and Lee 2003).

Another established procedure for the determination of vascular structure is the perfusion fixation technique. This procedure involves perfusion of a fixative solution through a fully relaxed vascular bed (Lee et al., 1983). Following histological preparation, the dimensions of the blood vessels can be measured. This technique has several advantages, including the avoidance of dissection retraction so that vascular structure is determined at *in vivo* length. CSA can be measured rather than calculated from measurements of lumen, media and wall thickness. Another advantage is that vascular density and rarefaction can be assessed in the perfusion-fixed tissue (Bund and Lee 2003). Disadvantages include the possibility of tissue shrinkage during the histological preparation, unless appropriate fixatives are used (Lee 1985). There is the possibility of incomplete relaxation during the application of a fixative. It is also difficult to achieve *in vivo* pressure using artificial perfusates, such as Krebs solution, because of the low viscosity of these solutions (Bund and Lee 2003). In the isolated hindquarter preparation of WKY rats, a flow rate of 30 ml/min/100 g body weight generated only 50 mm Hg of pressure, and such a high flow rate invariably resulted in vascular damage and oedema formation (Finch and Haeusler 1974). Lumen diameter also increases with the increase in perfusion pressure (Dickhout and Lee 2000). Another limitation is that the vessels are fixed

without vascular tone, so that the vascular dimensions under these conditions may not be exactly the same as those pertaining to the *in vivo* scenario (Bund and Lee 2003).

Several other authors tried to evaluate vascular morphological changes in hypertensive rats using non-perfusion methods. deBlois et al (1997) reported their method in vascular mass measurements using thoracic aorta from SHR. After rats were anesthetized the thoracic aorta was isolated and aortic media was snap frozen in liquid nitrogen and kept at -80°C until further processing. A 3 mm-long ring of aorta was cut and the aortic rings were fixed in 4% paraformaldehyde overnight and processed according to routine histological procedures for morphometric measurements in cross-sections of paraffin-embedded arteries. The medial cross-sectional area was evaluated in 5 μm-thick, hematoxylin-stained sections of aorta. Using this method, these authors reported successful assessment of the aortic hypertrophy in SHR that was further modulated by the administration of different antihypertensive drugs. They reported that the antihypertensive agents used in their study (Losartan, enalapril and nifedipine), which affected vascular hypertrophy, also stimulated smooth muscle cell apoptosis with a reduction in vascular DNA content and medial cell number as measured by the three-dimensional dissector method.

The aforementioned technique was adapted by several other researchers who used the same methodology but with modifications concerning staining or visualization using video-imaging (Der Sarkissian et al., 2004; Tea et al., 2000; Duguay et al., 2004). All these researchers used nonperfusion assessment of aortic vascular morphology in SHR before and after antihypertensive treatment and successfully confirmed their results by examining for various apoptotic changes in

the vasculature. Additionally, Zhu et al (2004) compared morphological parameters of mesenteric arteries from SHR and normotensive WKY rats and reported significantly higher media thickness/lumen thickness and media area/lumen area but lower lumen radius/vessel radius in SHR compared to age-matched WKY rats. SHR's vascular morphological changes were marginally reduced after antihypertensive treatment. These results were positively correlated with the mesenteric artery expression of transforming growth factor TGF-β1 and c-Jun mRNA. Dobrian et al (2000) reported significantly higher BP, arterial hypertrophy and a 2-fold increase in plasma renin activity in obese-prone SD rats compared with obese-resistant control rats.

Histological studies of the resistance vessels in hypertensive rats have the advantage of allowing global analysis, but may be compromised by unintended activation of vessels during fixation, and lack of knowledge of the intravascular pressure during the process, making none of the methods used perfect (Mulvany 2003). Nevertheless, if measurements are confined to a comparison of the ratio of wall thickness to lumen diameter 'wall:lumen' (or, with measurements of tunica media thickness, 'media:lumen'), ratios at a given lumen, all methods are in agreement that this parameter is increased in hypertension, at least in the more proximal resistance vessels (Mulvany 2003). The difficulty arises as soon as comparison is to be made of lumen diameters, or of the wall (media) cross-sectional areas, since here all of the methods are faced with the fundamental problem of comparing vessels from different individuals. Vascular architecture differs between individuals even of the same strain,

and substantial differences in architecture between strains have been documented (Schmid-Schonbein et al., 1986).

In the current study, the non-perfusion method for vascular morphology assessment was implemented. Data concerning lumen diameter, medial diameter and CSA obtained by this method demonstrated the presence of the "mostly agreed upon" eutrophic remodeling in the small peripheral arteries of adult SHR. Following hemin protocol, and with BP normalization of SHR, reversal of eutrophic remodeling changes was observed in the peripheral mesenteric vessels. No such changes were observed in the hemin-treated normotensive SD rats. Thus, the non-perfusion method was able to yield valid data concerning vascular morphological changes in this study.

One hypothesis for the long-lasting antihypertensive effect of hemin in SHR was that hemin released from subcutaneously implanted miniosmotic pumps may be accumulated in the implantation site. Absorption of hemin into the circulation from the hemin pump implantation site will lead to distribution of hemin to various organs and tissues, especially vascular tissues, where HO-1 expression is locally upregulated. After withdrawal of the hemin pumps, locally accumulated hemin may be gradually absorbed into circulation over several months so that HO-1 expression would have been continuously upregulated by hemin. Indeed, immediately after hemin pump implantation, serum heme level increased significantly. While this elevated serum heme level remained unchanged throughout the hemin pump implantation, it quickly returned to the basal level 2 weeks after hemin pump removal. Furthermore, at the time of animal sacrifice, either at the end of the 3-week hemin protocol or 9 months afterwards, no hemin accumulation was observed at the

subcutaneous implantation sites. We cannot, however, disregard the possibility that infused hemin may have accumulated in blood vessel wall locally and metabolized there slowly, which may have provided long-lasting stimulation of HO-1 upregulation.

There are several other explanations for the hemin protocol-induced long-lasting normalization of BP in adult SHR. Certainly, the observation that basal HO-1 expression in 12 week old SHR rats was at least twice as high as that seen in normotensive control, is of value. Hemin protocol was able to upregulate the expression and activity of HO in SHRs, but not SDs, for a long time afer treatment stoppage. It is then reasonable to suggest that SHR may inimitably have a hyper-reactive HO system that can respond, apparently in an irreversible way, to prolonged stimulation (Beierwaltes 2006).

The upregulated HO-1 expression during the 3-week hemin protocol is sustained even after the removal of hemin pump. This could be attributed to prolonged induction of HO-1 expression and/or prolonged stability of the existing HO-1 proteins, triggered by and maintained after 3-week hemin protocol. Hemin has been shown to activate gene expression and promote differentiation in a variety of cell types, including mouse 3T3 cells (Chen and London, 1981), neuroblastoma (Ishii and Maniatis 1978). These data suggest that hemin is a primary inducer of hemoglobin synthesis in pro-erythroid cells, an event consistent with the erythropoietin (EPO)-induced de novo hemoglobin synthesis during the early stages of hematopoiesis. Moreover, hemin has been shown to control the rate of total protein synthesis via a hemin-controlled repressor (London et al., 1981) and to selectively

activate the expression of both embryonic and fetal globin genes in human K-562 cells (Rutherford et al., 1979; Tsiftsoglou et al., 1989; Fibach et al., 1995).

More interestingly, and very relevant to our study, is that heme can activate both globin and HO-1 genes by binding to and removing the transcriptional repressor Bach1 (Sun et al., 2002). This means that heme suppresses Bach1's interaction with known DNA sequences, thus preventing its transcription repression action. It is therefore reasonable to suggest that 12 week old SHRs may have an already suppressed DNA transcriptional repressor activity of the HO-1 gene. The 3-week hemin protocol, with extensive and extended suppression of Bach 1, may have led to a long-term activation of HO-1 gene expression and HO activity. Consequently, this led to prolonged normalization of blood pressure observed in our study. However, it is still unclear from the available literature the mechanism through which heme binds to Bach1 and alters its conformation (Tsiftsoglou et al., 2006). Also, can hemin-induced suppression of Bach1 alter the expression of other genes that may have contributed, independent of HO activity, to the prolonged BP normalization observed in our study? This question needs to be addressed in the future.

The excessive and sustained high levels of HO activity led to a sustained decrease in VEGF expression in the small peripheral blood vessels in adult SHR 9 months after hemin pump removal. Low expression of VEGF suppressed eutrophic remodeling of small peripheral arteries in SHR. This structural change of vascular tissues would result in a relatively permanent reversal of hypertension.

Intravenously administered hemin has been recommended for the clinical treatment of acute intermittent porphyria (AIP) with a daily dosage of 3-4 mg/kg for 4

days (Anderson et al., 2005), which improved the conditions of AIP patients (McColl et al., 1981). Significant side effects of hemin therapy for porphyria have not been reported (Anderson et al., 2005) with only few cases of fever, aching, or malaise. In a clinical trial with 12 AIP patients, there were only 2 hypertension patients, female, aged 24 and 31 years (Lamon et al., 1979). After intravenous hematin injection (2-4 mg/kg/day) (Lamon et al., 1979), their BP was normalized 24 to 36 hours later. Serum heme concentration during the hematin injection period was between 2-6 mg/dl and dropped to undetectable baseline levels a few days after cessation of treatment. This normalized BP continued along with other symptomatic improvements after cessation of 4-9 days of hematin administration (Lamon et al., 1979). However, no follow up studies were conducted regarding the changes in BP level as well as AIP in these patients. It is also worth noting that these patients were normotensive until the emergence of AIP and their BP was restored after hematin administration. Therefore, the BP lowering effect of hematin in this study may be secondary to the improvement of AIP, rather than the upregulated HO-1 expression.

5.8 Safety profile of the hemin protocol

The hemin protocol through subcutaneously implanted pumps did not present itself as a toxic hazard. None of the hemin-treated animals exhibited growth retardation (Table 4.2). Liver damage usually leads to the release of ALT and γGT from the parenchymal cells into the blood. In this study, no significant changes in liver function tests were observed in any of the hemin-treated animals. Changes in

serum creatinine concentration more reliably reflect changes in glomerular filtration rate than do changes in serum urea concentrations (Kreder and Williams 2004). Serum urea and creatinine concentrations in the hemin-treated animals in our study remained in the normal range.

5.9 Effect of the 3-week hemin protocol on erectile response of adult SHR

Penile erection is known to be dependent upon the balance and integration between structural (vascular and extracellular matrix) and functional control systems (neural, local factors and humoral/ endocrine). The nature of the balance between these contributing influences is ultimately expressed as vasodilation and penile tumescence, as long as there is an adequate level of systemic BP and an appropriate hormonal milieu (Adams et al., 1997).

The penile vascular components that have a significant role in regulating erectile function are the pudendal arterial system, the cavernous arteries, the corpora cavernosa and the corpus spongiosum. There is usually one main supplying endartery per cavernosal body, which branches off from the corresponding internal iliac artery. This feeder artery branches to become the deep penile artery which has at least two types of branches within the cavernosa: helicine arteries which direct flow to lacunar spaces and vessels which direct flow for nutrient supply via capillaries (Adams et al., 1997).

It is well established that an erection requires a neurally mediated (autonomic) vasodilation of both the penile arterial blood vessels and the trabecular meshwork (Lue and Tanagho 1987). The combined dilatation facilitates an initial

rapid increase in arterial inflow into the cavernous bodies of the penis promoting tumescence. This is followed by a phase of decreased inflow resulting from the unstable phase involving the occlusion of sub-tunical veins and full rigidity, namely the veno-occlusive mechanism. Conversely, detumescence is likely mediated, at least in part, by activation of the sympathetic nervous system as well as removal of active vasodilator tone and in addition, may involve changes in local systems.

In men with hypertension, prevalence of ED is significantly higher than in the general population (Croog et al., 1988). In fact, 8–10% of untreated hypertensive patients are found to be suffering from ED once their hypertension is diagnosed (Lewis et al., 2000). The question has been raised as to whether the higher rate of sexual dysfunction in hypertensive individuals is the result of hypertension per se or due to antihypertensive medications (Behr-Roussel et al., 2003). Some authors suggested a possible causative role for some antihypertensive drugs such as diuretics and noncardioselective β-blockers (Behr-Roussel et al., 2003). Nevertheless, the functional and structural modifications induced by hypertension may also be strongly implicated in ED even though pathophysiological studies of ED induced by hypertension are surprisingly scarce (Chitaley et al., 2001).

Several arguments support the concept that pathophysiological modifications induced by hypertension itself may be implicated in the occurrence of ED. Among the hallmarks of hypertension are increased peripheral sympathetic activity (Head 1989), elevated vasoconstrictor tone, and decreased endothelium-dependent vasodilatations in arteries from both conductance and resistance vasculature (Taddei et al., 1997). The resulting imbalance in regulatory vascular tone

can either be linked to increased release of vasoconstricting and/or reduced release of dilating substances. In fact, the cyclooxygenase pathway has been identified as mainly involved in vascular endothelium-dependent alterations observed in hypertension (Kung and Luscher 1995). Interestingly, increased norepinephrine content has also been found in nonvascular tissue of SHR, implying that hyperinnervation is not restricted to the vasculature and may include other sympathetically innervated tissue, i.e., the genitourinary organs (Tong et al., 1996). Furthermore, hypertension also induces modifications in the vascular structure, i.e., remodeling that could participate in an overall reduced dilatory capacity (Folkow 1990). Interestingly, remodeling may also occur at the corporal level (Okabe et al., 1999) and thus participate in the pathophysiology of ED by altering the mechanical properties of the erectile tissue (Behr-Roussel et al., 2003).

In this study, it was firstly confirmed that the enormity of erectile responses was reduced significantly in adult SHR compared to age-matched normotensive SD rats. These results were in agreement with previous reports that the ICP was severely reduced in the plateau phase after electrical stimulation in adult SHR compared to age-matched WKY and SD rats (Behr-Roussel et al., 2003). This consistent phenomenon justifies the use of SHR as a reliable model for studying decreased erectile responses in hypertension. It is worth noting that the anesthetic combination of xylazine and ketamine used in these experiments was found in similar studies to have no significant effect on electrically-generated erectile responses in comparison to the frequently-used urethane (Behr-Roussel et al., 2003).

In the current study, a 21-day hemin protocol resulted in normalization of BP. This led to an interest in assessing the effect of this prolonged hemin protocol on ICP. Hemin protocol resulted in a surprising normalization of ICP in adult SHR. On the other hand, similar treatment duration of SHR with a well-known antihypertensive and direct smooth muscle relaxant, hydralazine, was able only to normalize BP but not ICP with no significant increase in cavernous HO-1 expression. It is thus reasonable to assume that the useful outcome of hemin on ICP is due to its specific effect on HO-1 expression and not merely due to normalized BP. While the basal expression of HO-1 was similar in both SHR and SD rats, the sensitivity of cavernous tissue of SHR to prolonged HO-1 inducer administration is much higher than that of SD.

In addition to the upregulated HO-1 expression, hemin protocol also increased the expression of sGC. The latter would elevate cellular level of cGMP. Increasing cGMP levels has been the target for many recent drugs to treat ED (Moreland et al., 2001). Phosphodiesterase (PDE) enzymes are widely distributed in tissues throughout the body. Mammalian PDEs comprise 11 identified families (PDE1 to PDE11), which are distinguished by their substrate specificities and tissue distributions (Rosen and Kostis 2003). Degradation of cGMP in the penile corpora is catalyzed primarily by PDE5 (Lue and Tanagho 1987). With high PDE5 levels, less cGMP will be available to stimulate PKG, resulting in increased intracellular calcium levels in smooth muscle cells of the helicine arteries and trabeculae of the cavernous tissue. Contraction of the cavernosal arteries and trabecular smooth muscle cells leads to a decrease in arterial penile blood inflow. With contraction of the corpus

cavernosum the intracavernosal pressure decreases and the stretch on the subtunical venules is reduced. Consequently, venous blood outflow from the penis increases, resulting in detumesence (Lue and Tanagho 1987). One of novel findings in this study is the significantly higher level of PDE5 in cavernous tissue of adult SHR, which could be a contributing mechanism to decreased erectile responses in these hypertensive rats. More interestingly, prolonged HO-1 upregulation normalized PDE5 levels in treated SHR, whereas cavernous tissue PDE5 levels in normotensive rats did not change significantly with hemin administration. It is hypothesized that prolonged stimulation of sGC in the cavernous tissue of SHR was coupled with decreased levels of abnormally high PDE5.

The present studies showed that decreased ICP was closely associated with increased BP levels. While hypertension was corrected by the hemin protocol, ICP responses in the treated SHR were normalized, too. Interestingly, hemin protocol in SHR resulted in a decrease in the erection onset, rather than complete elimination. It is postulated that with increased expression levels of sGC, any erectogenic stimulus (electric stimulation in this study) will rapidly increase the levels of cGMP in the cavernous tissue. This will lead to a greater and more rapid increase in the relaxation of the vascular smooth muscle cells in the cavernous tissue, resulting in a decrease in penile erection onset. Does high BP cause erectile dysfunction? Burchardt et al (2001) reported that ED might be a strong indicator of cardiovascular complications of hypertensive patients. On the other hand, Behr-Roussel et al (2003) suggested that BP level might not cause erectile dysfunction since penile pathologic morphological

changes occurred in young pre-hypertensive SHR, before the development of hypertension.

The present study clearly demonstrates that prolonged upregulation of HO-1 in cavernous tissue of hypertensive rats can lead to marked improvement in their abnormally low ICP. It is believed that these findings shed light, for the first time, on potential therapeutic strategies for ED through stimulation of the HO/CO system by HO-1 inducers.

6. Summary and Conclusions

Two important discoveries are reported in the present study. First, a 3-week hemin treatment of adult SHR can effectively lower high BP and lead to a prolonged and persistent normalization of BP for the following 9 months. These effects were mediated through sustained stimulation of the HO/CO system and its downstream effector targets. Increased acitivity of HO-1 led to normalization of the abnormally high levels of VEGF in the peripheral mesenteric arteries of adult SHR. Subsequently, this resulted in reversal of the eutrophic remodeling observed in these arteries, which, we believe was the prinicipal determinant of the long-term normalization of BP observed for 9 months after stoppage of hemin treatment.

Second, it was found that coupled to its remarkable effect on BP of adult SHR, hemin protocol led to normalization of the otherwise abnormally low intracavernous pressure. To our knowledge, no other antihypertensive agent can lead to normalization of high BP and low ICP present in hypertensive animals.

Results obtained from our work indicate the importance of HO/CO system in regulation of BP and ICP in SHR. It will also stimulate more research seeking to find more effective and long-term treatment of hypertension.

7. Future directions

1. *In vitro* studies are needed to investigate the effects of hemin protocol on the vascular contractility of resistance vessels. Specifically, whether hemin protocol can lead to altered endothelial-dependent and independent vascular responses needs to be examined. This could be done through applying acetylcholine, phenylephrine and sodium nitroprusside directly to isolated mesenteric arteries and examining for vascular response.

2. McClung et al (2004) suggested that HO-1 may have differential effects on cell-cycle proliferation in ECs and VSMCs. Following arterial injury, increasing HO-1 led to inhibition of VSMCs proliferation and neointima formation. Can the antiapoptotic effects of HO-1 on VSMCs proliferation be involved in the long-term BP normalization after hemin protocol? *In vivo* and *in vitro* studies investigating cell proliferation and apoptosis in the resistance arteries of adult SHR after hemin protocol are needed. This can be done through monitoring VSMCs' nuclear condensation and DNA fragmentation.

3. Hemin protocol was able to normalize high BP in adult SHR. SHR, an animal model of human hypertension, probably represents only one of many possible types of hypertension. Thus, to further understand the effects of hemin on BP regulation, hemin protocol should be tried on other types of animal models of hypertension. For example, the one kidney, one clip rat model represents a volume overload form of hypertension. The Dahl salt-sensitive rat is another model of hypertension combined

with heart failure. Other animal models of hypertension include the deoxcorticosterone acetate-salt and the Ang II-induced hypertension rat models.

4. Hemin protocol was able to normalize low ICP in adult SHR. However, the long-term effects of this erectile enhancing property of hemin were not examined. ICP of hemin-treated SHR should be monitored at different time points following termination of hemin protocol.

5. Aging is the most common underlying factor for the development of ED in men. In the current study, hemin protocol did not affect the ICP of adult normotensive rats. However, the effect of hemin protocol on old (>1 year) normotensive rats was not examined. Monitoring ICP after hemin protocol in old rats should be investigated.

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9. Appendix

Significant parts from chapters 2, 3 and 4 have been published in the following journals:

Shamloul R, Wang R. Monitoring circulatory heme level in hemin therapy for lowering BP in rats. Cell Mol Biol 2005;5:507-12.

Shamloul R, Wang R. Increased intracavernosal pressure response in hypertensive rats after chronic hemin treatment. Journal Sexual Medicine 2006;3:619-27.

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